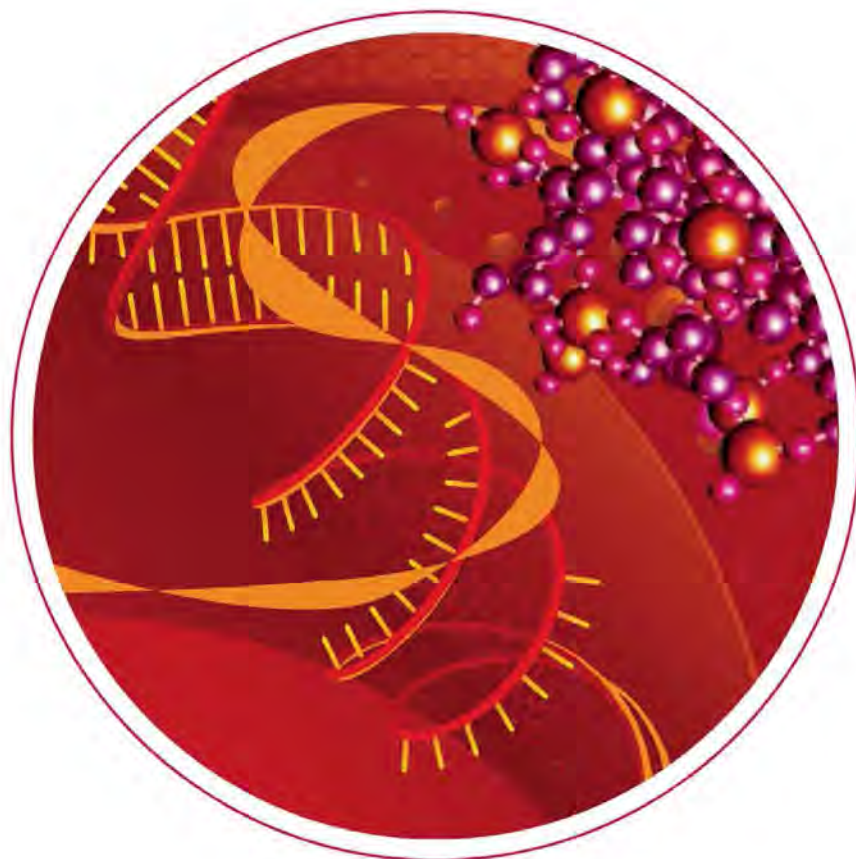


16 October 2009 \$10

Science

Advances in **Neuroscience Methods**



From Genes to Proteins:

The Impact of Gene Sequence on Translation and Expression

WEBINAR

Wednesday
October 28, 2009
12 noon Eastern;
9 a.m. Pacific;
4 p.m. GMT

Advances in life science technologies have enabled researchers to gain greater insight into the workings of our genetic code. This includes how subtle changes in gene sequences can impact the expression of encoded proteins through mechanisms including codon bias, mRNA stability, and translation initiation. Natural genes sequences have been shaped in response to many different evolutionary pressures, but are rarely optimal for aspects of “biotechnological fitness,” such as maximized protein yield or optimal expression control. In this webinar, our expert guests will discuss the current understanding of how and why gene coding sequences influence protein expression, and ways in which this understanding can help shape strategies to design genes for applications ranging from synthetic biology to protein crystallography.

Join our panel of experts in a live discussion. Register to participate.

Questions can be submitted live to the panel during the webinar or in advance via e-mail provided with registration. To register, visit

www.sciencemag.org/webinar

During the webinar, viewers will:

- obtain an overview of the current state of research in this field
- gain insight into the range of cellular processes affected by variations in gene sequence
- learn about the importance of optimizing gene sequences for numerous research applications
- have questions answered live by the panel of experts!

Participants:

Joshua Plotkin, Ph.D.
University of Pennsylvania
Philadelphia, PA

Christine Vogel, Ph.D.
University of Texas at Austin
Austin, TX

Mark Welch, Ph.D.
DNA 2.0
Menlo Park, CA

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SPECIAL SECTION

Neuroscience Methods

INTRODUCTION

- 385 So You Want to Learn How to Network?

NEWS

- 386 Alzheimer's Biomarker Initiative Hits Its Stride
Longitudinal Alzheimer's Studies Go Global
- 390 Massively Parallel Brain Imaging

REVIEWS

- 391 Molecular and Cellular Approaches to Memory Allocation in Neural Circuits
A. J. Silva et al.
- 395 The Optogenetic Catechism
G. Miesenböck
- 399 Modalities, Modes, and Models in Functional Neuroimaging
K. J. Friston

>> See also related Editorial p. 339 and Perspective p. 379

EDITORIAL

- 339 Great Expectations
Atsushi Miyawaki
>> Neuroscience Methods section p. 385

NEWS OF THE WEEK

- 346 Honors to Researchers Who Probed Atomic Structure of Ribosomes
>> Science Express Research Articles by *T. M. Schmeing et al.* and *Y.-G. Gao et al.*;
Science Express Perspective by *A. Liljas*
- 347 Laureates Analyzed Economics Outside Markets

- 349 Enzyme Lets You Enjoy the Bubbly
>> Report p. 443
- 349 From Science's Online Daily News Site
- 350 Tying Up the Solar System With a Ribbon of Charged Particles
>> See the six IBEX-related Science Express Reports
- 350 In Holland, the Public Face of Flu Takes a Hit
- 351 From the Science Policy Blog
- 352 Tonegawa Rethinks Japan's Premier Brain Research Center
- 353 Russian Expats Challenge Government's 'Disastrous' Support for Science
- 353 Lunar Mission: A Slam, But Was It a Dunk?

NEWS FOCUS

- 354 The Big Gamble in the Saudi Desert
>> Science Podcast
- 358 Fetal Cells Again?
- 361 A Guru of the Green Revolution Reflects on Borlaug's Legacy
>> Perspective p. 381
- 362 From Burning Dung to Global Warming and Back Again

LETTERS

- 364 Turning Ivory Towers into a Golden Economy
T. Kaufman and T. Katsouleas
Bushmeat Hunting as Climate Threat
J. F. Brodie and H. K. Gibbs
Climate Engineering Vulnerable to Disruption
C. Leovy
Conserve Livestock Genetic Resources, Too
P. J. Boettcher and I. Hoffmann
Teaching, Not Testing, for Scientific Vision
R. Root-Bernstein
- 366 CORRECTIONS AND CLARIFICATIONS



page 354

BOOKS ET AL.

- 368 Darwinian Populations and Natural Selection
P. Godfrey-Smith, reviewed by J. Odenbaugh
- 369 The Elusive Malaria Vaccine
I. W. Sherman, reviewed by B. Greenwood

POLICY FORUM

- 370 Balancing Innovation and Access: Patent Challenges Tip the Scales
M. J. Higgins and S. J. H. Graham

PERSPECTIVES

- 372 The Speaking Brain
P. Hagoort and W. J. M. Levelt
>> Report p. 445
- 373 Becoming *T. rex*
J. Clark
>> Report p. 418
- 374 A Ball-and-Chain Polymer Model
C. J. O. Reichhardt and L. M. Lopatina
>> Report p. 408
- 375 Observing Monopoles in a Magnetic Analog of Ice
M. J. P. Gingras
>> Reports pp. 411 and 415

CONTENTS continued >>



COVER

A frontal view of the fly brain (blue) showing two groups of dopamine-producing neurons pseudocolored green and magenta. The magenta neurons are engineered to express a light-sensitive protein. Optical signals (symbolized by a magenta beam of light) can selectively report and control the activity of these cells. Miesenböck (page 395) reviews the emerging field of optogenetics in a special section on advances in neuroscience methods starting on page 385.

Confocal images: Adam Claridge-Chang;
photomontage: Robert Roorda and Gero Miesenböck

DEPARTMENTS

- 335 This Week in Science
- 340 Editors' Choice
- 342 Science Staff
- 345 Random Samples
- 457 New Products
- 458 Science Careers

- 377 **Fantastic Fixers**
R. W. Fulweiler
>> Report p. 422
- 378 **Feeding the Clock**
D. M. Suter and U. Schibler
>> Report p. 437
- 379 **How Good Are Neuron Models?**
W. Gerstner and R. Naud
>> Neuroscience Methods section p. 385
- 381 **Retrospective:**
Norman Ernest Borlaug (1914–2009)
C. Dowsell
>> News story p. 361

ESSAY

- 382 **Eppendorf Winner: Evolution and Revolution in Odor Detection**
R. Benton

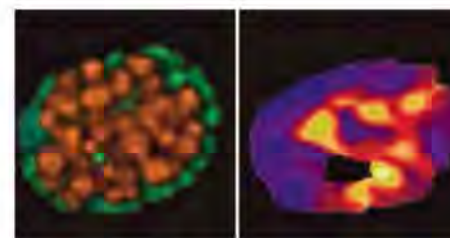
BREVIA

- 404 **Direct Evidence for Spinal Cord Involvement in Placebo Analgesia**
F. Eippert et al.
Functional magnetic resonance imaging of the human spinal cord reveals a mechanism for placebo analgesia.

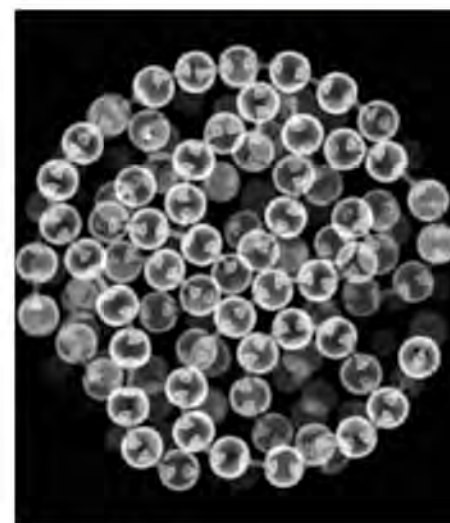
REPORTS

- 405 **Phase Transitions, Melting Dynamics, and Solid-State Diffusion in a Nano Test Tube**
V. C. Holmberg et al.
A carbon coating allows the thermodynamic behavior of a germanium nanowire to be probed under constant-volume conditions.
- 408 **The Packing of Granular Polymer Chains**
L.-N. Zou et al.
The packing of connected metal rods is used as a model to study the packing behavior of granular materials and polymers.
>> Perspective p. 374
- 411 **Dirac Strings and Magnetic Monopoles in the Spin Ice $\text{Dy}_2\text{Ti}_2\text{O}_7$**
D. J. P. Morris et al.
- 415 **Magnetic Coulomb Phase in the Spin Ice $\text{Ho}_2\text{Ti}_2\text{O}_7$**
T. Fennell et al.
Neutron scattering measurements on two spin-ice compounds show evidence for magnetic monopoles.
>> Perspective p. 375
- 418 **Tyrannosaurid Skeletal Design First Evolved at Small Body Size**
P. C. Sereno et al.
The distinct features of *Tyrannosaurs*, such as their large skull and tiny arms, appear in an earlier small-bodied species.
>> Perspective p. 373

- 422 **Deep-Sea Archaea Fix and Share Nitrogen in Methane-Consuming Microbial Consortia**
A. E. Dekas et al.
Methane-oxidizing bacteria in marine sediments may also be a major factor in ocean nitrogen cycling.
>> Perspective p. 377
- 426 **Generation of Functional Ventricular Heart Muscle from Mouse Ventricular Progenitor Cells**
I. J. Domian et al.
A combination of tissue engineering and stem cell biology is used to build functional force-generating mouse cardiac tissue.
- 430 **Generation of Medaka Fish Haploid Embryonic Stem Cells**
M. Yi et al.
Stem cells that are haploid can sustain stable growth, pluripotency, and genetic integrity in fish cell cultures.
- 433 **Complete Resequencing of 40 Genomes Reveals Domestication Events and Genes in Silkworm (*Bombyx*)**
Q. Xia et al.
Silkworm genomes show signatures of selection associated with domestication.
- 437 **AMPK Regulates the Circadian Clock by Cryptochrome Phosphorylation and Degradation**
K. A. Lamia et al.
The protein kinase AMPK couples circadian clocks and metabolism in mammals through effects on a cryptochrome protein.
>> Perspective p. 378
- 440 **Teachers' Participation in Research Programs Improves Their Students' Achievement in Science**
S. C. Silverstein et al.
Students of U.S. high-school teachers given science research experiences show improved success rates on science exams.
- 443 **The Taste of Carbonation**
J. Chandrasekar et al.
The enzyme carbonic anhydrase mediates the taste sensation of carbonated drinks.
>> News story p. 349; Science Podcast
- 445 **Sequential Processing of Lexical, Grammatical, and Phonological Information Within Broca's Area**
N. T. Sahin et al.
Intracranial electrodes record activity in a language-associated area of the brain as words are identified and produced.
>> Perspective p. 372
- 449 **Fast Synaptic Subcortical Control of Hippocampal Circuits**
V. Varga et al.
A form of subcortical control of cortical information processing is mediated by a synaptic release of serotonin and glutamate.



pages 377 & 422



pages 374 & 408



page 430

CONTENTS continued >>

SCIENCEONLINE

SCIENCEEXPRESS

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Global Observations of the Interstellar Interaction from the Interstellar Boundary Explorer (IBEX)

D. J. McComas et al.

10.1126/science.1180906

>> *Science Podcast*

Width and Variation of the ENA Flux Ribbon Observed by the Interstellar Boundary Explorer

S. A. Fuselier et al.

10.1126/science.1180981

Structures and Spectral Variations of the Outer Heliosphere in IBEX Energetic Neutral Atom Maps

H. O. Funsten et al.

10.1126/science.1180927

Comparison of Interstellar Boundary Explorer Observations with 3D Global Heliospheric Models

N. A. Schwadron et al.

Observations by the Interstellar Boundary Explorer have revealed surprising features in the interaction between the heliosphere and the interstellar medium.

10.1126/science.1180986

Direct Observations of Interstellar H, He, and O by the Interstellar Boundary Explorer

E. Möbius et al.

Detection of H, He, and O flowing into the heliosphere from the interstellar medium tells us about our local interstellar environment.

10.1126/science.1180971

Imaging the Interaction of the Heliosphere with the Interstellar Medium from Saturn with Cassini

S. M. Krimigis et al.

Observations by Cassini show that some of the features revealed by IBEX extend to high energies.

10.1126/science.1181079

>> *News story p. 350*

PTP α Is a Receptor for Chondroitin Sulfate Proteoglycan, an Inhibitor of Neural Regeneration

Y. Shen et al.

Mouse neurons that lack a receptor for inhibitory proteoglycans show improved regeneration.

10.1126/science.1178310

The Crystal Structure of the Ribosome Bound to EF-Tu and Aminoacyl-tRNA

T. M. Schmeing et al.

10.1126/science.1179700

>> *Science Podcast*

The Structure of the Ribosome with Elongation Factor G Trapped in the Posttranslocational State

Y.-G. Gao et al.

Crystal structures of the ribosome bound to elongation factors provide insights into translocation and decoding.

10.1126/science.1179709

>> *Science Podcast*

Leaps in Translational Elongation

A. Liljas

10.1126/science.1181511

>> *News story p. 346*

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Highlights From Our Daily News Coverage

Physics? It's All the Same to Birds and Babies

Rooks can grasp a basic physical principle, but how smart does that make them?

How We Lost Our Diversity

Human ancestors survived two genetic bottlenecks as they spread out of Africa.

Monkey Moms Have Madonna Moments

Rhesus macaques oogle their babies just like human mothers do.

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RESEARCH ARTICLE: MicroRNAs Differentially Regulated by Akt Isoforms Control EMT and Stem Cell Renewal in Cancer Cells

D. Iliopoulos et al.

Akt-dependent induction of a metastatic phenotype may depend on the balance of Akt1 and Akt2.

RESEARCH ARTICLE: Act1, a U-box E3 Ubiquitin Ligase for IL-17 Signaling

C. Liu et al.

PERSPECTIVE: IL-17 Receptor Signaling—Ubiquitin Gets In On the Act

S. D. Levin

The adaptor protein Act1 functions as a ubiquitin ligase to mediate interleukin-17 receptor-dependent activation of nuclear factor- κ B.

RESEARCH ARTICLE: Chemical Genetics Identifies c-Src as an Activator of Primitive Ectoderm Formation in Murine Embryonic Stem Cells

M. A. Meyn III and T. E. Smithgall

PODCAST

M. A. Meyn III and A. M. VanHook

Kinases engineered for inhibitor resistance reveal a unique role for c-Src in embryonic stem cell differentiation.

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Perspective: Three Crucial Questions When Applying to M.D.-Ph.D. Programs

L. F. Brass

Here are key factors to consider when deciding if an M.D.-Ph.D. is right for you.

Finding Your Way Into Policy Careers in Europe

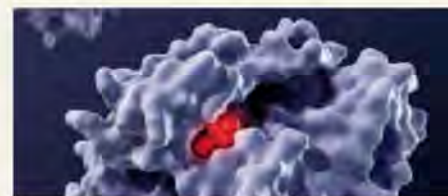
E. Pain

Getting a policy job in Europe requires choosing a beat and finding your way in.

Tooling Up: Focus Your Industry CV

D. Jensen

Small changes in your CV can yield big rewards.



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Engineering inhibitor-resistant kinases.

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RESEARCH: Dopamine Gene Therapy for Parkinson's Disease in a Nonhuman Primate Without Associated Dyskinesia

B. Jarraya et al.

PERSPECTIVE: Gene Therapy for Dopamine Replacement in Parkinson's Disease

A. Björklund et al.

Delivery of the synthetic enzymes for dopamine to Parkinsonian monkeys in a single vector ameliorates disease symptoms.

PERSPECTIVE: Arvid Carlsson, An Early Pioneer in Translational Medicine

J. K. Andersen

Carlsson discovered the L-dopa treatment for Parkinson's.

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A History of Beginnings

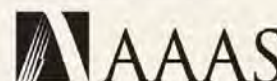
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Science Policy News and Analysis

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ADVANCING SCIENCE. SERVING SOCIETY

Seeing the Brain's One, Two, Three >>

Taking advantage of the rare opportunity to record neuronal activity in the human brain using intracranial electrodes, **Sahin et al.** (p. 445; see the Perspective by **Hagoort and Levelt**) document the spatial and temporal pattern of neuronal populations within Broca's area as patients thought of a single word, changed its tense (for verbs) or number (for nouns), and articulated the word silently. For these three stages, they detected activity at 200, 320, and 450 milliseconds, moving in a caudal to rostral direction. These data fit neatly within the roughly 600 milliseconds required for the onset of speech and map the distinct neural computations within an area of the brain, known for almost a century and a half, as important for the production of language.



Confined Germanium Thermodynamics

When a material is confined to nanoscale volumes, the very high proportion of surface to bulk can alter its thermodynamic properties. This has been studied using in situ electron microscopy, but in most cases the volume of the material is not constrained. **Holmberg et al.** (p. 405) studied the thermodynamics of a germanium nanowire attached to a gold seed and coated with a carbon shell to restrict its volume, measuring the reaction temperature, as well as the liquid composition without changes in volume throughout the heating cycle. This enabled monitoring of phase behavior while the germanium was being heated, and tracking solid-state diffusion across the confined interface.

Balancing the Nitrogen Budget

Setting the global budget for elements presents difficult challenges, such as accounting for possibly unknown sources or sinks. An unresolved imbalance in the oceanic nitrogen budget suggests that there may be additional sources of biological nitrogen fixation in the deep sea. Using high-resolution imaging techniques, **Dekas et al.** (p. 422; see the Perspective by **Fulweiler**) observed direct assimilation of isotopically labeled N_2 by anaerobic methane-oxidizing archaea from deep marine sediment and the subsequent transfer of nitrogen to their sulfate-reducing bacterial symbionts. This slow and energetically costly conversion by archaea is

dependent upon methane and requires physical contact with the associated bacterial partner. Such syntrophic consortia represent a potential source of nitrogen in the oceans and may help to balance the global nitrogen budget.

Like Beads on a String

The optimal packing of spheres, and the somewhat lower densities obtained in the compaction of granular materials are well studied problems. What is less clear is what happens when the spheres are connected, as in the case of polymeric materials—often represented by connected sphere models. **Zou et al.** (p. 408; see the Perspective by **Reichhardt and Lopatina**) examined the packing of chains of metal beads commonly used for securing bathroom drain plugs or for raising or lowering window blinds. Both the length of the chains, and whether they were linear or looped, influenced the overall packing density. Jamming the chains together captured the key physics of the glass transition of polymeric materials.

Magnetic Monopoles

Magnets come with a north and a south pole. Despite being predicted to exist, searches in astronomy and in high-energy particle physics experiments for magnetic monopoles (either north or south on their own) have defied observation. Theoretical work in condensed-matter systems has predicted that spin-ice structures may harbor such elusive particles (see the Perspective

by **Gingras**). **Fennell et al.** (p. 415, published online 3 September) and **Morris et al.** (p. 411, published online 3 September) used polarized neutron scattering to probe the spin structure forming in two spin-ice compounds— $Ho_2Ti_2O_7$ and $Dy_2Ti_2O_7$ —and present results in support of the presence of magnetic monopoles in both materials.

Haploid Medaka Stem Cells

Although diploid embryonic stem cells have been generated by various means, there would also be value in deriving haploid stem cells. In these cells, recessive mutations in essential genes would show phenotypes that would not be apparent in heterozygous animals. **Yi et al.** (p. 430) used the medaka fish model system to generate haploid stem cells that show stable growth and pluripotency. In addition, a fertile female medaka fish was produced by haploid embryonic stem cell nuclear transfer into a normal egg. This system has potential for analyzing recessive genes, for example, in disease phenotypes or in various cell lineages in culture.

Diddy Dinosaurs

Tyrannosaurs were the dominant large dinosaur predator during the Late Cretaceous. They have several distinct specialized features, including an oversized skull, huge hindlimbs, and tiny arms, that have been thought to have evolved in concert with their large size and carnivorous diet.



Sereno et al. (p. 418, published online 17 September; see the Perspective by **Clark**) now describe an earlier, diminutive tyrannosaur from China that also has these common specializations. Thus, these features were not a result of size increase but appear to have been required for feeding efficiency at all sizes.

Coupling Clocks and Metabolism

Circadian clocks in mammals coordinate behavior and physiology with daily light-dark cycles by driving rhythmic transcription of thousands of genes. The master clock in the brain is set by light, but clocks in peripheral tissues, such as the liver, are set by daily feeding. Such coupling should allow tissues to "anticipate" food

Continued on page 337

Continued from page 335

consumption and optimize the timing of metabolic processes, but how nutritional status is communicated to peripheral clocks is unclear. Studying cell culture models and mice, **Lamia *et al.*** (p. 437; see the Perspective by **Suter and Schibler**) show that the nutrient-responsive signaling molecule AMPK (AMP-activated protein kinase) provides metabolic information to circadian clocks by triggering phosphorylation and subsequent degradation of the clock component cryptochrome-1. Thus—cryptochromes, which originally evolved as blue-light photoreceptors in plants, act as chemical energy sensors in mammals.

The Taming of the Silkworm

Silkworms, *Bombyx mori*, represent one of the few domesticated insects, having been domesticated over 10,000 years ago. **Xia *et al.*** (p. 433, published online 27 August) sequenced 29 domestic and 11 wild silkworm lines and identified genes that were most likely to be selected during domestication. These genes represent those that enhance silk production, reproduction, and growth. Furthermore, silkworms were probably only domesticated once from a large progenitor population, rather than on multiple occasions, as has been observed for other domesticated animals.

Teaching Teacher

High-school science teachers rarely have an opportunity to participate in scientific research. A program from Columbia University studies what happens when these teachers do get laboratory experience. After a summer program spanning 2 years, including professional development programs, as well as laboratory research, **Silverstein *et al.*** (p. 440) show that the teachers' students benefit. Students of teachers who participated in this summer research experience showed greater pass rates on the tough New York State Regents exams than did students of teachers who did not participate in the program.

A Fix to the Heart

Regenerative cardiovascular medicine is a promising avenue for therapeutic application in advanced heart failure. Although clinical trials have suggested some limited benefits in cell transplantation therapy, robust cardiac muscle formation is lacking. **Domian *et al.*** (p. 426) examined the developmental processes in normal mature cardiac muscle. A two-color murine reporter system was used to isolate committed ventricular progenitors, which were then used to build functional force-generating cardiac tissue. Such combinations of tissue engineering and stem cell biology may eventually lead to cardiac regenerative therapy.



Gee Fizz

The next time you enjoy a carbonated beverage, you can do so with an enhanced understanding of the molecular mechanism that provides its distinctive flavor sensation. **Chandrashekar *et al.*** (p. 443) genetically ablated specific sets of taste cells in mice and found that the sensation of CO₂ was lost in animals lacking taste cells that sense sour flavors. A screen for genes specifically expressed in these cells revealed the gene encoding carbonic anhydrase 4, which catalyzes hydration of CO₂ to form bicarbonate and free protons. Knockout animals not expressing the carbonic anhydrase 4 gene also showed diminished sensation of CO₂. The protons produced by the enzyme appear to be the actual molecules sensed by the sour-sensitive cells. This process, combined with tactile sensations, appears to be the source of the popular fizzy sensation.

Subcortical Network Regulation

Subcortical neuromodulatory centers dominate the motivational and emotional state-dependent control of cortical functions. Control of cortical circuits has been thought to involve a slow, diffuse neuromodulation that affects the excitability of large numbers of neurons relatively indiscriminately. **Varga *et al.*** (p. 449) describe a form of subcortical control of cortical information processing whereby strong, spatiotemporally precise excitatory input from midbrain serotonergic neurons produces a robust activation of hippocampal interneurons. This effect is mediated by a synaptic release of both serotonin and glutamate and impacts network activity patterns.

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Atsushi Miyawaki is a director of the Advanced Technology Development Core at the RIKEN Brain Science Institute, Saitama, Japan, and a research director of the ERATO Life Function Dynamics Project of the Japan Science and Technology Agency. E-mail: matsushi@brain.riken.jp

Great Expectations

A CONVERGENCE OF DIVERSE DISCIPLINES IS FUELING THE EXPANSION OF NEUROSCIENCE RESEARCH, and underpinning this growth are increasingly advanced technologies such as high-performance computation, simulation and neuron modeling, real-time and high-resolution brain imaging, advanced recording techniques, and detailed neuron mapping. This special issue of *Science* gives a well-deserved nod to the technological innovations that have improved our understanding of the development, function, and disorders of the nervous system, and that will continue to open up many new questions about the human brain.

Today, there are many state-of-the-art institutions worldwide that focus on brain and cognitive science, helping to bridge the gaps between different knowledge bases, such as those centered on genomic information or brain images. Ten years after its establishment in 1997, the RIKEN Brain Science Institute reorganized to establish an Advanced Technology Development Core, designed to create new methods for studying the brain. Our efforts will hopefully speed the progress of neuroscience as we address the growing needs of this field. For example, more robust means are needed to characterize brain signals in ways that will enhance the ability to monitor neural processes. There is also a need for tools that detect the dynamic organization and coordinated activity of populations of neurons in the brain, so as to explore their relevance to behavior and cognition. And we want to approach information processing through new mathematical tools, to better understand how ensembles of neurons process information and consequently modify their structures as the brain learns. As well, genetic engineering approaches must be further refined if we are to analyze the neuronal circuits that underlie complex conditions such as mental disorders.

One approach that is becoming increasingly important combines optical and molecular genetic methods. These “optogenetic” technologies facilitate the discovery of mechanisms by which neurons process and integrate synaptic inputs. Genetically encoded sensors, constructed by fusing fluorescent proteins to functional proteins involved in physiological signaling, now allow the use of light to image excitable cell activity in large sets of neurons. These sensors can be introduced into neurons selectively, and they extract neuronal signals from an intact brain more efficiently than can organic dyes. By limiting the expression of a sensor to a subpopulation of neurons, one can visualize the connectivity between multiple different neuron groups and examine how the activity of specific neurons contributes to the function of neural circuits. Different sensors have thus far been developed to investigate signal integration, synaptic transmission, and neuronal plasticity; we’re only in the very early stages of exploiting this method to its full potential.

Ultimately, we want to understand brain function in a physiological context. In this regard, a key approach to complement these combined optical-genetic methods involves generating animals to which two-photon excitation microscopy can be applied. This microscopic approach has markedly increased penetration into tissue, and it is being widely used to view the intact brain. Because of recent progress in gene transfer techniques, including virus-mediated gene transfer and germline transmission of transgenes, the experimental animals to be studied are not limited to mice and can be extended to nonhuman primates.

In addition, these new genetically encoded tools are likely to spark an evolution in microscopy. For example, advances in brain imaging have enabled scientists to use miniaturized fluorescence microscopes based on fiber optics to observe brain activity in awake behaving animals. A new microscopy system will also be needed for the large-scale reconstruction of neuronal circuits in brain samples, because one major goal of the neuroscience community is to create a complete physical map of the human nervous system. Soon we may be admiring sweeping views of axons and dendrites as they carry signals that travel long distances.

Our expectations for neuroscience discovery remain high. With technologies continually improving, why shouldn’t they be?

— Atsushi Miyawaki



EDUCATION

Questions, Questions



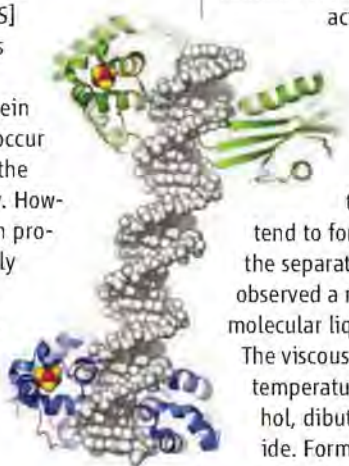
Question-asking is a fundamental element of practicing science; however, the teaching of question-asking skills is often overlooked in science education. Practicing scientists appreciate the value of asking questions, but do students in science classes? Keeling *et al.* asked undergraduate students in a senior-level cell biology class to review background material, detailed protocols, types of data to be recorded, and guidelines for analysis, before their laboratory session. After reflecting on the scientific process behind upcoming experiments, students were asked to write at least three questions about the science to be studied. As might be hoped, students' questioning ability, as assessed by the quality and relevance of questions generated, improved over time. Although repeated practice at writing questions did lead to improvement, "learning by doing" may not be enough, and specifically teaching question-asking skills could usefully be paired with explicit guidance and discussion. — MM

Life Sci. Edu. 8 131 (2009)

MOLECULAR BIOLOGY

Resolving DNA Repair

Base excision repair (BER) enzymes involved in fixing damaged DNA have low specificity for their targets and occur in low numbers in cells. So how are DNA damage sites located on chromosomes with any efficiency? Boal *et al.* suggest that the key lies in DNA charge transport chemistry, so that repair proteins can signal to each other rapidly over long distances. The ordered stacking that occurs in DNA makes it an excellent medium for electron transport, and the process is very sensitive to the presence of mismatched bases. When a BER enzyme containing an [4Fe4S] cluster binds DNA it becomes oxidized. In the absence of damage, when a second protein binds, electron transfer will occur and the reduced member of the protein pair will diffuse away. However, if there is damage, both proteins remain bound and, fairly rapidly, repair proteins will redistribute to the damaged region. Boal *et al.* used atomic force microscopy to establish that redistribution did take place in complexes of the BER enzyme EndoIII with DNA duplexes containing a sin-



DNA with bound repair proteins.

gle mismatch. As predicted, cooperation was observed between different BER enzymes, the bacterial EndoIII and MutY. These enzymes have human homologs linked to cancer predisposition, although the medical implications of the conservation of these iron-sulfur clusters remain unclear. — BJ

Proc. Natl. Acad. Sci. U.S.A. 106, 15237 (2009).

CHEMISTRY

A Charge from Sulfur

A zwitterion is essentially a pair of oppositely charged ions connected by a covalent bridge. A common example is the structure of an amino acid dissolved in water near neutral pH—the amino group strips a proton from the carboxylic acid group, leading to a positive ammonium and a negative carboxylate. In general, these highly polarized structures tend to form in solvents that can stabilize the separated charges. Heldebrant *et al.* have observed a rare instance in which a pure molecular liquid adopts zwitterionic character. The viscous yellow substance forms at room temperature on exposure of an amino alcohol, dibutylundecanolamine, to sulfur dioxide. Formally, the sulfur bonds to the terminal oxygen of the alcohol, forming a negatively charged sulfite group, while the proton formerly on that oxygen shifts to the opposite

terminus, forming a positively charged ammonium center. The authors characterized the unusual liquid spectroscopically and discovered that the SO₂ addition is reversible. Raising the temperature under reduced pressure leads to release of the gas and recovery of the amino alcohol precursor. Because the precursor does not react with CO₂, the SO₂ binding and release cycle might be applicable to selective removal and recovery of sulfur from combustion exhaust streams. — JSY

Energy Environ. Sci. 2, 10.1039/b916550a (2009).

BIOMEDICINE

Great Expectations

Two genetic mutations that in combination cause cell death, whereas each alone does not, are known as synthetically lethal. Fairly recently, the synthetic lethality notion has been applied to the search for anticancer drugs, to identify lethal combinations of drugs with specific tumorigenic mutations. The first targeted anticancer drugs to emerge from this approach were inhibitors of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). These drugs exploit the synthetically lethal interaction between PARP and the tumor suppressor genes *breast cancer 1* (*BRCA1*) or *BRCA2*, which are involved in DNA double-strand break repair by homologous recombination. Mutations in the *BRCA* genes are associated with an increased risk of breast and ovarian cancer, and PARP inhibitors are currently showing great

promise in the clinic. Another tumor suppressor gene, *phosphatase and tensin homolog (PTEN)*, is one of the most frequently mutated genes in human cancers and has been implicated in genome stability. Now, Mendes-Pereira *et al.* have found a role for PTEN in homologous recombination, and they demonstrate the sensitivity of PTEN mutant tumor cells to PARP inhibitors. PTEN-deficient cells were also highly sensitive to PARP inhibitors. BRCA mutations are relatively infrequent and limited mainly to breast and ovarian cancers. This study suggests that PARP inhibitors may also be useful in a far broader range of cancers that involve PTEN mutations, such as lung and colorectal tumors and glioblastomas. — HP

EMBO Mol. Med. **1**, 315 (2009).

DEVELOPMENT

Macho Mice

Male-specific behaviors in mice include mating, aggression, and territorial marking. Development of male-specific behaviors in mice, as well as in humans, requires the testicular hormone testosterone. The hormone estrogen, better known for its function in females, is also required. In males, which lack ovaries, estrogen is produced when the enzyme aromatase converts testosterone to estrogen. Estrogen produced by aromatase-expressing cells in the brain then signals through receptors to affect behavior. Wu *et al.* have now analyzed the neurons that express aromatase and their connectivity in mice. Although aromatase-expressing neurons represent only a minority of the neurons in the brain, the locations and patterns of these neurons in the adult mouse brain reflect sexual dimorphism. Sexually dimorphic territorial behaviors are also affected by disruption of estrogen-based signaling. The testosterone surge around the time of birth in mice thus seems to direct masculinization of territorial behaviors through aromatization of testosterone into estrogen in key neurons within the brain, rather than through androgen receptor signaling. — PJH

Cell **139**, 61, (2009).

APPLIED PHYSICS

What a Whopper

The basic design of a laser sandwiches a gain medium between two mirrors. As the light bounces between the mirrors, the intensity of the electromagnetic field builds up within the cavity and spills out from one of the mirrors.

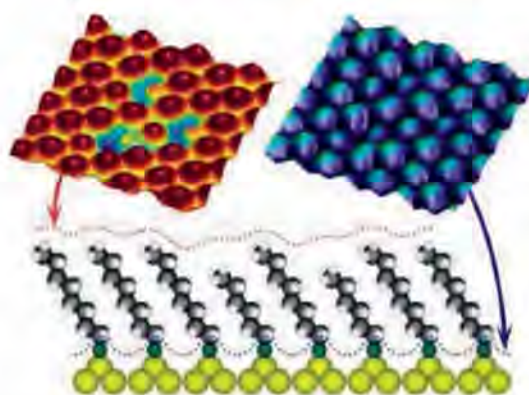
Laser instrument size can vary greatly, from the tiny semiconductor devices in DVD and CD players, to the tabletop-confined systems used in spectroscopy and other laboratory applications. Then there are the football field-sized instruments used to probe high-energy light/matter interactions in attempts to harness the class of fusion energy released by the Sun. Reaching an even greater size scale, Turitsyn *et al.* present a fiber laser with a cavity 270 kilometers long. Such an ultralong laser could be used in secure communications. Because the properties of the waves building up within the medium depend on how the light bounces off the mirrors, a given protocol between a sender and receiver for manipulating the mirrors could provide mode selection for securely relaying messages. — ISO

Phys. Rev. Lett. **103**, 133901 (2009).

CHEMISTRY

Imaging Top and Bottom

Scanning tunneling microscopy of adsorbed molecules on surfaces usually interrogates the topography of the topmost layer of atoms. For small molecules, this information is often sufficient to answer many structural questions, but for long-chain molecules, the bonding of the molecule to the surface can be obscured, espe-



cially at high surface coverages. Han *et al.* imaged both the topmost layer and the bonding interface of alkane thiols containing up to 10 carbon atoms adsorbed on gold. The application of an ac modulation of the tunneling gap allowed the buried methyl groups at the gold/sulfur interface to be imaged in a derivative mode. In the high coverage regime, the coexisting domains of different packing arrangements varied in their gold/sulfur bonding motifs in a manner consistent with previous x-ray diffraction studies. This technique will also be useful in studying other processes where changes in molecular tilt impact function. — PDS

ACS Nano **3**, 10.1021/nn901030x (2009).

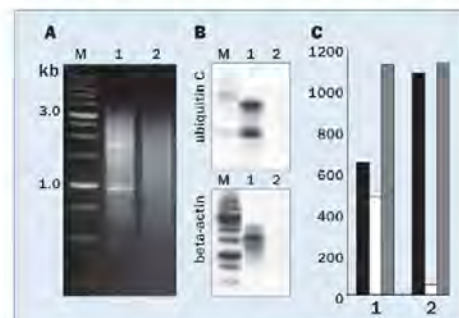
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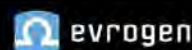
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Typical cDNA normalization result.

(A) Agarose gel electrophoresis of cDNA samples; (B) Virtual Northern blot analysis of abundant transcripts in these cDNA samples; (C) Sequencing of randomly picked clones: black columns – unique, white columns – non-unique, grey columns – all sequences. 1 – non-normalized cDNA; 2 – normalized cDNA. M – 1 kb DNA size markers.

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Pretty Poison

Swine flu can be beautiful. Below is one of the large glass "viral sculptures" that were on show this month at a London gallery. Artist Luke Jerram has also rendered HIV, smallpox, SARS, and *Escherichia coli* in glass with advice from virologist Andrew Davidson of the University of Bristol in the United Kingdom. The renditions are more faithful to the originals than most illustrations, Jerram says, because they are naturally transparent. More images are at http://www.lukejerram.com/projects/glass_microbiology.



Bumper Crop

Net-wielding entomologists have captured and named only a small fraction of the world's insects. Now researchers have come up with a new strategy: Drive through them and study the DNA stuck to your bumper.

The "Galaxy Team," a multiuniversity group of biologists and computer experts (<http://galaxyproject.org>) took two road trips, from Pennsylvania to Connecticut and then from Maine to New Brunswick, Canada. They attached sticky tape to their front bumper to collect everything airborne along the way. Back at the lab, they extracted DNA from the splatter and compared it with a database of DNA from known species.

The exercise yielded more than 400,000 different DNA fragments from as many as 2000 insect species as well as innumerable species of bacteria. Only 8% of the fragments matched known sequences. Most of the rest were likely from microbes, plants, and animals poorly represented in the database, the team reported online last week in *Genome Research*. The identified sequences reflected the difference in biodiversity between two regions, one urbanized and the other heavily forested.

The technique of analyzing DNA from the environment, known as metagenomics, has been used to study hard-to-culture microorganisms such as bacteria in seawater but never bug-sized creatures. The "radiator approach" is a quick-and-dirty method, says entomologist Donald Chandler of the University of New Hampshire, Durham, "but it seems to do the job."



NIH LOSES AN ICON

Ruth Kirschstein, 82, former deputy director of the National Institutes of Health, died on 6 October at the NIH Clinical Center in Bethesda, Maryland. Kirschstein's history is interwoven with that of NIH. She and her husband, Alan Rabson, now a deputy director at the National Cancer Institute, came to Bethesda in the 1950s to work as pathology researchers. In 1974, she became the first woman to direct an NIH institute, the National Institute of General Medical Sciences. Kirschstein served as NIH deputy director under Harold Varmus and held the fort for 29 months as acting director before Elias Zerhouni took over. "She knew everything, everybody, every rule and was an incredible resource," says Varmus.

Despite poor health, Kirschstein was still working until last week, NIH Director Francis Collins said in an e-mail to staff. "The world has lost one of its dearest, most dedicated public servants, one with a huge heart and brilliant mind," Collins said.

Hoards and Hordes

Buried coins may provide clues about population fluctuations in the Roman Republic more than 2000 years ago, scholars say.

Historians have long debated Rome's population in the first century B.C.E. when the republic fell. Censuses begun in 28 B.C.E. by the first Roman emperor, Augustus, pegged the empire's population at about 5 million—10 times as many as a century earlier.

What happened? Social scientists Peter Turchin of the University of Connecticut, Storrs, and Walter Scheidel of Stanford University in Palo Alto, California, took coin hoarding—which increases in times of peril—as a proxy for social instability. First they compared data on coin burials with population fluctuations between 250 B.C.E. and 100 B.C.E. and found that hoarding jumped during the Second Punic War, when the population dropped by 50,000. Then they inferred later population levels from data on coins hoarded between 100 B.C.E. and 50 C.E.

They concluded that the population of adult males then was about half the Augustan census count. So these censuses must have been counting women and children, they reported online last week in the *Proceedings of the National Academy of Sciences*. True or not, "This paper has the great virtue of pushing the debate back toward actual evidence," says historian Ian Morris of Stanford University.



Pot of 10,000 coins found in Shrewsbury, U.K.

CHEMISTRY NOBEL

Honors to Researchers Who Probed Atomic Structure of Ribosomes

Just as architects usually get more glory than carpenters do, DNA is more famous than the molecular machine that converts genetic blueprints into proteins. But the ribosome was in the limelight last week with the announcement of this year's Nobel Prize in chemistry.

The prize was awarded to three scientists who revealed the atomic structure and inner workings of the ribosome: Ada Yonath of the Weizmann Institute of Science in Rehovot, Israel; Thomas Steitz of Yale University; and Venkatraman Ramakrishnan of the Medical Research Council Laboratory of Molecular Biology in Cambridge, U.K. All three used a technique known as x-ray crystallography to pinpoint the position of thousands of atoms in the ribosome, and each will share one-third of the \$1.4 million prize.

"It's a fantastic accomplishment and one that everyone in the field has known for some time is worthy of such recognition," says Wayne Hendrickson, an x-ray crystallographer at Columbia University. But Hendrickson and others—including all three new Nobelists themselves—note that the Nobel Committee's self-imposed rule limiting each prize to no more than three recipients left other ribosome pioneers out in the cold.

"There were several scientists who contributed at least as much as the three of us," Yonath says. Among those most often mentioned: Harry Noller of the University of California (UC), Santa Cruz, who, among other things, led the group that first solved the crystal structure of the complete ribosome; Peter Moore, a ribosome biochemist and Steitz's colleague at Yale; and Joachim Frank at Columbia, whose electron microscopy studies proved crucial in work-

ing out ribosomal structures. "It was an impossible situation to acknowledge them all," says Jamie Cate, a structural biologist and ribosome expert at UC Berkeley.

Ribosomes have sparked such scientific interest because they are the final major player in biology's central dogma, which describes how in all cells the genetic information in DNA is first translated into messenger RNA and then converted by ribosomes into proteins. To make that happen, dozens of different proteins and strands of RNA form a complicated machine divided into two principal components. The smaller

the arrangement of atoms in the molecule.

In 1980, Yonath created the first low-quality crystals of the 50S subunit. By 1990, she had upped the quality of her crystals, but she still struggled to map a good structure. Steitz and Moore jumped into the fray in 1995, following Yonath's recipe for making ribosomal crystals. In 1998, they used additional insights gleaned from electron microscopy studies to acquire a low-resolution 9-angstrom structure of the 50S subunit. In August 2000, Steitz's group increased the resolution to 2.4 angstroms (*Science*, 11 August 2000, p. 905). Yonath's and Ramakrishnan's groups published slightly lower resolution structures of the smaller subunit the following month.

Since then, the three groups and others have begun to combine these atomic snapshots, and others like them, into a jerky movie of the atomic dance ribosomes perform to

translate genetic information into proteins. Structural biologists have captured dances of complex molecules before. The ribosome's dance, however, is more like a grand ballet, with dozens of ribosomal proteins and subunits pirouetting with every step while other key biomolecules leap in, carrying other dancers needed to complete the act.

In a pair of research articles published online this week in *Science* (www.sciencemag.org/cgi/content/abstract/1179700 and 1179709), for example, Ramakrishnan and colleagues reveal in atomic detail the choreography of how two key cocatalysts—abbreviated EF-Tu and EF-G—swoop into the heart of the ribosome carrying amino acids, drop them off, and toss them to another site where they are tacked onto the end of the growing protein chain. Steitz calls Ramakrishnan's new work "very good" and confesses to some envy, as his own group toiled for years trying to achieve the same feat.

Still, there are plenty of insights to come, Steitz and others say. So far, all known ribosomal crystal structures have



Puzzle masters. Ramakrishnan (left), Steitz, Yonath, and others pieced together the ribosome's structure, which contains hundreds of thousands of atoms.

component, known as the 30S subunit, works mainly to decode the genetic code in messenger RNA. The larger 50S subunit then takes this information and uses it to stitch together amino acids in the proper sequence to make up the final protein.

Early researchers struggled to map the atomic structure of even one of these subunits. Producing an x-ray structure requires first creating crystals of millions of copies of a ribosome aligned in near-perfect order. Researchers then fire a beam of x-rays at the crystal. If the ordering of the crystal is precise enough, the pattern in which those x-rays deflect off the crystal can be used to map out



Saudi Arabia's
big venture

354



Gearing up for
fetal cell transplants

358

come from prokaryotes. Researchers are racing to generate the first atomic-level structure of a eukaryotic ribosome, which are more complex and less uniform than those from prokaryotes. Yonath adds that her group is closing in on another long-standing goal: identifying what could be a "proto-ribosome," a set of highly conserved RNAs within modern ribosomes that may have formed the original catalytic center for

forging the peptide bonds that link amino acids together. If true, this proto-ribosome could add major support to the "RNA-world hypothesis," which suggests that life originally evolved with its genetic blueprint encoded in RNA.

The three groups have also begun to push practical applications of their work. All three, for example, have reported crystal structures that show how different anti-

biotics bind to the ribosome. Several companies are now using these structures in an effort to design new antibiotics against worrisome infections, such as methicillin-resistant *Staphylococcus aureus* and tuberculosis. As these antibiotics and other advances continue to roll in, ribosomes figure to remain in the spotlight for many years to come.

—ROBERT F. SERVICE

ECONOMICS NOBEL

Laureates Analyzed Economics Outside Markets

Last year, financial markets took the worst drubbing since the Great Depression, so perhaps not surprisingly this year's "Nobel Prize" in economics honors two researchers who studied economic behavior in other settings. Half of the 2009 Sveriges Riksbank Prize in Economic Sciences in Memory of Alfred Nobel goes to Elinor Ostrom, a political scientist at the University of Indiana, Bloomington, for her insights into the use of shared resources. The other half of the \$1.4 million prize honors Oliver Williamson, an economist at the University of California, Berkeley, for his analysis of how a company decides what to do or make for itself and what to buy from others.

"The really great thing is that this [year's prize] recognizes that we should look at the institutions that govern economic activity, which include a lot of things that aren't markets," says Robert Gibbons, an economist at the Sloan School of Management at the Massachusetts Institute of Technology in Cambridge.

Ostrom, 76 years old and the first woman to win the economics prize, has made her career studying how people cooperate to manage a common resource. For example, all users of a fishery can benefit by limiting their catches to prevent overfishing. But self-interest can torpedo cooperation, as each fisher can maximize profits by hauling in as much as possible. That "tragedy of the commons" seems to be an inevitable consequence of rational self-interest.

Economists had consequently believed that cooperation had to be imposed by the

government or induced by market incentives. And yet, beginning with fieldwork on efforts to halt saltwater intrusion into groundwater in southern California for her doctorate in 1965, Ostrom found that some communities do share communal resources. Through case studies, theoretical analysis, and experiments, she identified seven principles that foster cooperation. "Work on the commons is almost

Williamson, 77, studied how a company decides which goods and services to provide for itself and which ones it will buy—decisions that delimit the "boundary of the firm," explains Gibbons. "At one time, Henry Ford was investing in rubber plantations in South America," he says. "Now car companies buy tires."

Such decisions involve a balancing of benefits and costs. Suppose a sewing machine company uses a specialized part made by only one supplier. The company could save money by making the part itself, especially if the lone supplier inflates prices. On the other hand, if the company makes things that it could buy from one of several competing suppliers, it may waste money.

Williamson's analysis serves both as a description of how companies naturally behave and as a prescription for how to organize a company, says Francine Lafontaine, an economist at the Ross School of Business at the University of Michigan, Ann Arbor. "I don't think there's an economist out there who doesn't know Williamson's name," she says. "There's no question he deserves this recognition."

Nonmarket economics may also get more recognition, researchers predict. Close alliances between megabusineses such as Wal-Mart and their suppliers are putting a new wrinkle on the boundary of the firm, Gibbons says. And Gächter says that climate change looms as the ultimate problem—and perhaps tragedy—of the commons.

—ADRIAN CHO



Broader vision. Williamson (left) and Ostrom studied institutions other than markets.

synonymous with Ostrom," says Simon Gächter, an economist at the University of Nottingham, U.K. "She's a towering figure in the literature."

Ostrom found that individuals will cooperate if, among other things, they are able to participate in governance, monitor the compliance of others, and punish cheaters. "When people have trust that others are going to reciprocate, then there can be cooperation," she says. "When there is no trust, there is no cooperation unless people are facing the gun."

ScienceNOW.org

NEUROSCIENCE

Enzyme Lets You Enjoy the Bubbly

In 1988, a curious letter to the editor appeared in *The American Journal of Medicine*. One of the two co-authors, a physician named Stephen Kelleher, had recently climbed a mountain while taking the drug acetazolamide, commonly used as a prophylactic against altitude sickness. The climbing party had brought along a six-pack of beer, envisioning a celebratory drink at the summit. But the brew tasted flat, “like dishwater,” the letter lamented. Further experimentation “in the interest of science” revealed that acetazolamide also ruined the taste of carbonated soda but not whiskey. Noting that a previous study, published in Norwegian, had made similar observations, Kelleher and his co-author Mark Graber dubbed this effect the champagne blues.

On page 443, a team headed by Charles Zuker, a neuroscientist now at Columbia University, helps explain this puzzling phenomenon. Using methods borrowed from electrophysiology and genetic engineering, the researchers identified a class of taste-receptor cells in the tongue that respond to carbon dioxide (CO₂), the gas that gives sparkling beverages their fizz. They also report that the molecular sensor used by these cells to detect CO₂ is an enzyme called carbonic anhydrase 4—one member of the class of enzymes inhibited by acetazolamide.

“It’s a genetic validation of the champagne blues,” says Stephen Roper, a neuroscientist at the University of Miami, Florida. Roper says the new work confirms and extends earlier evidence that carbonic anhydrases are responsible for CO₂ perception, and it shows for the first time that the fizz-sensing cells on the tongue are the same taste-receptor cells that detect sourness.

Historically, many researchers assumed that the tingling sensation of carbonated beverages arises because the bursting of CO₂ bubbles stimulates mechanoreceptors in the mouth, says Zuker. But some observations, including those of the mountaineers, suggested that chemoreceptors on the tongue—such as those responsible for detecting sweet,

salty, sour, bitter, and umami—also played a role, Zuker says.

His team, which specializes in taste receptors, began tackling this bubbly debate by recording neural activity in the main nerve from the taste-receptor cells of the tongue in mice given a taste of club soda or exposed to CO₂ gas. Both increased neural firing. Next, they repeated this experiment in mice genetically modified to ablate specific populations of taste-receptor cells. Mice that lacked sour-sensing taste cells exhibited no neural responses to carbonation, indicating that without these cells they didn’t detect CO₂.

When the researchers then identified genes expressed only in the sour-sensing cells, one stood out. Called *Car4*, the gene encodes a carbonic anhydrase, which converts CO₂ into bicarbonate ions and free protons. Applying a drug that inhibits carbonic anhydrases substantially reduced, but didn’t totally eliminate, the mouse neural responses to CO₂. The same was true in mice engineered to lack the *Car4* gene. The team concludes that *Car4*, which sits in the membrane of sour-sensing cells, is the primary chemoreceptor for CO₂. Zuker says the free protons created when *Car4* breaks down CO₂ probably stimulate the sour-sensing cells just as free protons from an acidic food or drink would.

So why don’t carbonated beverages always taste sour? Zuker isn’t sure, but he speculates that the brain interprets input from the sour-sensing cells differently when it receives simultaneous input from the mechanoreceptive cells that respond to bursting CO₂ bubbles. At the same time, he notes there is some evidence that carbonation can be perceived as sour: In German, for example, seltzer water can be called sauer Sprudel or Sauerwasser.

Sadly, though, the work brings us no closer to a cure for the champagne blues. As Graber and Kelleher wrote more than 20 years ago, “Mountaineers must choose to leave either their acetazolamide, or their suds, at home.”

—GREG MILLER

From Science’s
Online Daily News Site

Physics Is All the Same to Birds and Babies

Even with their tiny bird brains, rooks comprehend basic principles of physics at the same level as a 6-month-old baby—and beyond that of chimpanzees—a new study reports. But whether this understanding conveys any advantages remains an open question. <http://bit.ly/119b78>

Monkey Moms
Have Madonna
Moments

It’s a look that’s been painted and photographed untold times: a mother gazing deep into her infant’s eyes while the two smile and kiss. Now, scientists have discovered similarly intense shared gazing and facial expressions in monkeys. And that, the researchers say, means that this kind of maternal communication dates back at least 30 million years. <http://bit.ly/tNqk3>

Famous Royals Suffered
From Hemophilia

Many of Queen Victoria’s male descendants died from a condition that caused them to bleed to death. This “royal disease” spread as Victoria’s heirs married into royal families across Europe, decimating the thrones of Britain, Germany, Russia, and Spain. Now, new DNA analysis on the bones of the last Russian royal family, the Romanovs, indicates that the royal disease was a rare subtype of hemophilia known as hemophilia B. <http://bit.ly/1Anpng>

How We Lost Our Diversity

If you compare any two people from far-flung corners of the globe, their genomes will be much more similar than those of any pair of chimpanzees, gorillas, or other apes from different populations. Evolutionary geneticists have now shown that our ancestors lost much of their genetic diversity in two dramatic bottlenecks that sharply squeezed down the population of modern humans as they moved out of Africa between 60,000 and 50,000 years ago. <http://bit.ly/ASIVU>

Read the full postings, comments, and more on scienown.sciencemag.org.

SPACE PHYSICS

Tying Up the Solar System With a Ribbon of Charged Particles

Talk about rewriting the textbooks. Scientists thought they knew what the heliosphere looked like. The solar system's enveloping pocket filled with the solar wind's charged particles is plowing through the onrushing "galactic wind" of the interstellar medium in the shape of a comet, they all agreed. When NASA launched the Interstellar Boundary Explorer (IBEX) in October 2008, mission scientists expected it to confirm the textbooks.

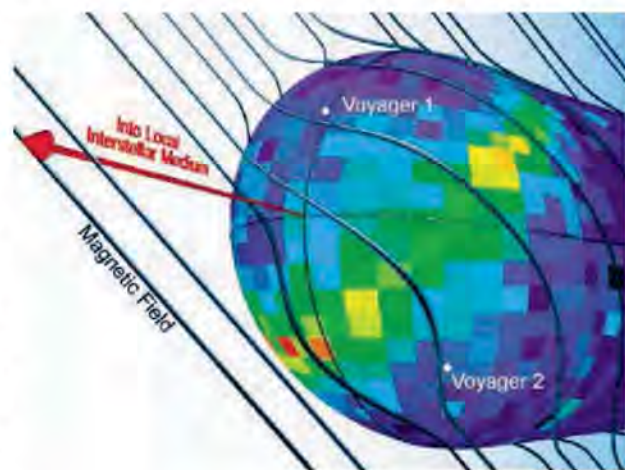
Instead, five papers published online in *Science* this week (www.sciencemag.org/cgi/content/abstract/1180906, 1180981, 1180927, 1180986, 1180971) report that IBEX revealed a sky-spanning "ribbon" of unexpectedly intense emissions of energetic neutral atoms (ENAs). No one knows what is creating the ENA ribbon, but everyone agrees that it means the textbook picture of the heliosphere is wrong. "I'm blown away completely," says space physicist Neil Murphy of NASA's Jet Propulsion Laboratory in

Pasadena, California. "It's amazing, it's opened up a new kind of astronomy."

The meterwide, hexagonal IBEX monitors the edge of the solar system from Earth orbit by "seeing" the heliosphere's outer boundary in the "light" of energetic neutral hydrogen atoms. They're energetic

because they are fast and neutral because they were once positive ions that lost their charge by picking up electrons. Those energetic parent ions lie near the heliopause, the boundary where solar wind meets galactic wind more than 100 times Earth's distance from the sun. Charged ions can't reach Earth, buffeted as they are by the solar wind and its embedded magnetic field. But once an energetic ion picks up an electron from a neutral atom, it can fly straight and true just like light. IBEX's two ENA detectors can then record the ENA glow from every direction.

"The thing that's really shocking is this ribbon," says IBEX principal investigator David McComas of Southwest Research Institute in San Antonio, Texas. Researchers had expected gusts in the solar wind blowing against the boundary to create 20% or 30% variations in ENA emissions, but the ribbon is 10



The squeeze is on. A ribbon of energetic neutral atoms (reds to greens) mark where the galactic magnetic field compresses the heliosphere.

VIROLOGY

In Holland, the Public Face of Flu Takes a Hit

AMSTERDAM—For the past 6 months, one could barely switch on the television here without seeing the face of famed virus hunter Albert Osterhaus talking about the swine flu pandemic. Or so it has seemed. Osterhaus, who runs an internationally renowned virus lab at Erasmus Medical Center in Rotterdam, has been Mr. Flu. But last week, his reputation took a nosedive after it was alleged that he had been stoking pandemic fears to promote his own business interests in vaccine development. As *Science* went to press, the Dutch House of Representatives had even slated an emergency debate about the matter.

So far, few incriminating facts have emerged, and Osterhaus has the backing of his employer as well as Dutch Health Minister Ab Klink, to whom he is an adviser. Yet the media frenzy has cast a pall on Osterhaus's public image that may be difficult to undo, and some say it undermines the already flagging confidence in the safety of flu vaccines and the motives of those promoting them.

It's no secret that Osterhaus, a workaholic

with more than 700 scientific papers to his name, also loves cameras and microphones (*Science*, 23 May 2003, p. 1228). But recently, some fellow scientists have accused Osterhaus of fear-mongering. Luc Bonneux, an epidemiologist at the Netherlands Interdisciplinary Demographic Institute, says Osterhaus is part of a "flu mafia" and that he's hyping the threats of both avian and swine flu. Miquel Ekkelenkamp, a microbiologist at University Medical Center Utrecht, called Osterhaus a "panic virologist" in a recent op-ed, adding that he "should be banned from television permanently."

The current firestorm erupted when reports surfaced that Osterhaus is co-founder of, and owns a 9.9% share in, ViroClinics, a Rotterdam spinoff from his lab that is involved in vaccine development. Fleur Agema, a member of the House of Representatives for the Party of Freedom, has said that's irreconcilable with Osterhaus's role on a Health Council panel that advises the Dutch government on flu vaccination.

Osterhaus says ViroClinics isn't itself

developing a vaccine against swine flu; it provides services—such as testing for antibodies in specimens—for a range of companies that do. His warnings about the pandemic peril don't affect ViroClinics' bottom line, says Osterhaus, because vaccine companies would have developed a vaccine against the new virus—and given his company business—no matter what. His company doesn't make more money if more vaccine is sold, he stresses.

Erasmus Medical Center agrees, but the Health Council sees it slightly differently. Osterhaus is "a little too close" to industry to be a voting member on the panel, says Executive Director Anneke Wjbenga; to avoid even the appearance of a conflict of interest, he has the status of an "adviser." Wjbenga stresses that there's nothing untoward about this situation, however, and that Osterhaus has been transparent about his financial interests. A Health Council statement also praises Osterhaus as "the absolute world top" in his field and says "we are glad ... to make use of his expertise." Health Minister Klink echoed that point of view in a letter to parliament. But

CREDIT: ADAPTED FROM D. J. MCCOMAS ET AL./SCIENCE

times that intense—a narrow band blazing across the sky like some Milky Way on fire. Charged particles have apparently become bunched along the ribbon near the boundary, says McComas, but how they got there “is still a big mystery.”

Researchers do agree that the ribbon requires abandoning the textbook comet shape for the heliosphere. “We’ve learned from IBEX that [the comet shape] is not quite right,” says McComas. In the old view, the solar wind dominated the heliosphere, creating an ideal head while the tail swept downstream in the galactic wind. But when he and colleagues compare IBEX data with simulations, McComas says, they see signs that the magnetic field lines of the galactic wind impinge on the heliosphere, pushing it inward where the magnetic pressure is greatest. That also appears to be where the ENA ribbon forms. In the IBEX group’s best model, the strong pressure from outside creates a misshapen but still recognizable heliosphere.

Space physicist Stamatios Krimigis of The Johns Hopkins University Applied Physics Laboratory in Laurel, Maryland, sees a radically different picture. In another online paper (www.sciencemag.org/cgi/content/abstract/1181079), he and col-

leagues report that they found an ENA ribbon in observations made by the Cassini spacecraft orbiting Saturn, at somewhat different energies from those IBEX detects. But Krimigis thinks the external magnetic forces of the galactic wind dominate. “There is some sort of ‘head,’ but it’s not the dominant effect,” he wrote in an e-mail, and there is “no ‘tail’ per se.” The heliosphere is more of a bubble, he says, squashed a bit by the galactic wind with solar wind escaping both upwind and downwind. “It looks more like a bubble of gas that wends its way through the galactic magnetic field,” he adds.

Sorting out the heliosphere’s true shape will take more time, says Murphy: “The geometry’s tough.” The shape is no doubt somewhere between the two extremes of ideal comet and pure bubble, but all agree that researchers will have to understand how the ribbon forms to know the heliosphere’s true shape. A key to that understanding, says Murphy, will be “how the fingerprint of the solar wind [is] seen to evolve” in the ENA observations as the sun moves toward the maximum of its 11-year cycle. Another key will be a lot more ribbon modeling.

—RICHARD A. KERR

Bonneux says that even as an adviser, “an alpha male like Osterhaus is going to dominate such a committee.”

Bonneux has also taken aim at the European Scientific Working Group on Influenza (ESWI), an industry-supported group that Osterhaus has chaired since 2000. ESWI organizes meetings, promotes awareness about flu, and tries to raise vaccination levels. Bonneux says the group—which receives a €40,000 contribution from each of 10 companies annually—is little more than a lobby club that gives “trumped-up, sensational stories a scientific seal of approval.” ESWI spokesperson Derek Smith, a flu scientist at the University of Cambridge in the United Kingdom and a close collaborator of Osterhaus’s, dismisses the claim, pointing out that ESWI’s independence is guaranteed by a Code of Practice.

Eelko Hak, a clinical epidemiologist at the University Medical Center Groningen who also sits on the Health Council panel,



Sidekick. Albert Osterhaus (right) is an adviser to Dutch Health Minister Ab Klink (left) and often joins him at news conferences on flu.

says that although the Dutch government encourages scientists to create spinoffs and ties with industry, popular opinion is still very uncomfortable with that idea. “It’s typically Dutch to make such a fuss about this,” he says. Hak worries about the fallout from the affair; the Netherlands has always had very high vaccination rates, but a recent campaign against the human papillomavirus, which causes cervical cancer, flopped because of public distrust of health authorities. The Osterhaus brouhaha is another blow, says Hak. —MARTIN ENSERINK

ScienceInsider

From the Science Policy Blog



French authorities last week charged a nuclear physicist working as a contractor at CERN with having ties to an Algerian terrorist organization. <http://bit.ly/2cn84q>

Fifty-one academic biomedical scientists in Spain published an open letter in *El País* declaring their “confusion” at plans for a €5.3 billion science and innovation budget, a 0.2% increase over this year’s budget. <http://bit.ly/2eS1Lm>. They are also worried that the government’s new commitment to industrial research would reduce funding for grants. <http://bit.ly/10KNqZ>

Marcia McNutt and Arun Majumdar sailed through a Senate nomination hearing for positions as head, respectively, of the U.S. Geological Survey and the Energy Department’s Advanced Research Projects Agency for Energy. <http://bit.ly/6jR3M>

The U.S. Centers for Disease Control and Prevention is urging people to get vaccinated against the H1N1 virus, which has killed 76 U.S. children. It also released results that suggested seasonal flu shots will not make people more susceptible to H1N1. <http://bit.ly/4igbgM>

The U.S. patent office has dropped rules proposed by the Bush Administration to reduce paperwork by limiting the number of follow-on patent applications allowed for an invention. The patent bar, which had sued the agency over the proposal, declared victory, but reform advocates said that the proposed rules were a good idea. <http://bit.ly/fE5Tw>

Congress has held up money to start building the \$450 million National Bio and Agro-Defense Facility in Manhattan, Kansas, because of concerns about the safety of local residents. The money was left out of a bill funding the Department of Homeland Security as lawmakers asked to see results of further studies before providing any construction money. <http://bit.ly/17WRud>

For more science policy news, visit blogs.sciencemag.org/scienceinsider.



Lab bench quarterback. As he shakes up BSI, Susumu Tonegawa will continue doing research at MIT.

NEWSMAKER INTERVIEW

Tonegawa Rethinks Japan's Premier Brain Research Center

WAKO, JAPAN—Susumu Tonegawa, 70, has never shied away from challenges. He left Japan to earn a Ph.D. in molecular biology from the University of California, San Diego. After a postdoc at the Salk Institute for Biological Studies, also in San Diego, he joined the Basel Institute for Immunology in Switzerland, where he solved the riddle of how mammals produce billions of different antibodies needed to fend off infections—work that earned him the Nobel Prize in physiology or medicine in 1987.

Tonegawa was then at the Massachusetts Institute of Technology (MIT) in Cambridge, where he shifted his focus to neuroscience and in 1994 became the founding director of what is now called the Picower Institute for Learning and Memory at MIT. After a 2006 flap over the aborted hiring of a young female scientist at another MIT institute, Tonegawa gave up the directorship to concentrate on research (*Science*, 24 November 2006, p. 1227). But this April, he became director of the RIKEN Brain Science Institute (BSI) in Wako, near Tokyo, a part-time arrangement that allows him to maintain a lab at MIT.

BSI was established in 1997 and now has more than 50 principal investigators (PIs) and a \$100 million annual budget. Considered Japan's flagship neuroscience institute, BSI is "pretty good," Tonegawa says, but it doesn't match the reputation and productivity of top U.S. and European neuroscience

centers. Tonegawa spoke with *Science* earlier this month about how he intends to raise BSI's game while coping with what he views as an inevitable downsizing.

—DENNIS NORMILE

Q: What are your challenges at BSI?

S.T.: Three major things. One is [rethinking the] direction of research. Second is [reforming] the system of principal investigator recruitment and promotion. And the third is to maintain good funding from the government and possibly other sources.

Q: Do you intend to maintain BSI's broad research agenda or narrow the focus?

S.T.: [Founding Director Masao] Ito felt he had to cover everything related to the brain: understanding the brain, protecting the brain, creating the brain, and nurturing the brain. It was very broad, ... partially as a strategy for justifying this large amount of government funding. My view is that that's too broad. This institute is going to be run with my bias of what I think is important for the future. I would like to put more emphasis on understanding the brain. Also, BSI has been quite strong in disease studies. I want to continue to promote that.

Also, we have to think about broadness versus concentrated research [because of] funding. In the early years, it was a tremendously rich institute. Now I don't think we can avoid some reduction [in the budget]. Gradually, the total number of labs will be reduced.

Q: What are the most interesting questions in neuroscience right now?

S.T.: The most interesting question is to try to understand circuits and the circuit basis of cognition. On one side, molecular and cellular studies have advanced tremendously. On the other side, there are very high-level studies in cognitive sciences, using [techniques such as] functional magnetic resonance imaging. The excitement for the coming decades will be to work out the events and processes that happen in between. We now have new technologies and enough knowledge to study the function of specific neural circuits that play a crucial role in behavior and cognition. And then you want to study which specific circuit goes wrong in neurodegenerative and psychiatric diseases.

Q: BSI introduced a system of rigorous reviews of each PI every 5 years. Has that worked, and are you going to modify that approach?

S.T.: I'm modifying it. A 5-year review repeated two times, three times, four times, I find, is silly. I'm introducing a tenure system. For the 22 or 23 people who have passed two reviews already, we've been requesting they get 15 or 16 letters from authorities throughout the world to decide whether they will be offered tenure. If they pass, they don't need to be reviewed anymore until they reach 65, when reviews will be reintroduced.

I don't want scientists to occupy space and use up funding without being productive. In the U.S., it becomes more difficult to acquire grants. Here, with internal grants, I want to look at the person's productivity every 3 years and come up with a reasonable agreement to reduce space and funding. It is possible some people will continue to work at full scale; it will depend on the person. When we hire young PIs, we will give them 4 to 6 years to demonstrate that they are very viable scientists. If they don't get tenure, they have to leave within 2 years.

Q: Can you do justice to this job splitting your time between here and MIT?

S.T.: I cannot leave research; that's my driving force for being alive. My research is at MIT—I don't have a lab here. When I come here, I spend all of my time running the place. Many daily things I delegate [to four associate directors]. Of course, it would be better if [a director] was here all the time. But in that case, it would have to be somebody who can do this job with great enthusiasm and motivation without having a lab.

CREDIT: D. NORMILE/SCIENCE

SCIENCE POLICY

Russian Expats Challenge Government's 'Disastrous' Support for Science

MOSCOW—On 2 October, 100 Russian researchers who permanently work abroad published a letter in the leading Moscow business newspaper *Vedomosti* complaining of “the disastrous situation in Russian basic research.” The letter, addressed to Russia’s president and its prime minister, pointed in particular to extremely low levels of funding and a continuing massive brain drain. “We certainly hope to draw the attention of the political leadership of the country to the dangers of neglecting fundamental science and education,” says Andrei Starinets, a physicist at the University of Oxford in the United Kingdom and an author of the letter. “It takes years before investments in fundamental science and education pay off. These issues therefore require strategic rather than tactical thinking.”

The letter hasn’t drawn any formal response so far, but Russian officials last

week boasted about their support of science, particularly a new program to lure back 100 expat researchers to work at least 2 months a year in a Russian research institute or university. “The process of return of researchers to Russia will become avalanche-like in the nearest future,” predicted the minister of science and education, Andrey Fursenko, at the Second International Nanotechnology Forum held in Moscow last week.

Starinets and the other letter-signers suggested that the country try to attract world-scale scientific projects, using as a concrete example the construction of the International Linear Collider. This high-energy particle collider, envisioned as a possible successor to the Large Hadron Collider, would boost Russia’s research in many fields, including information science, biology, and materials science, they say.

Russian researchers within the country haven’t all embraced the letter and its desire for big-science projects. Mikhail Gelfand, deputy director of the Kharkevich Institute for Information Transmission Problems, says that although more money for science would be good, “it would not give any result without radical reforms in science.” He also took issue with the suggestion to launch big projects, particularly to build a next-generation collider in Russia, suggesting that such projects would drain money from more worthwhile projects.

Starinets notes that the letter was circulated for signatures only to scientists who have permanent positions abroad, to avoid any criticism that support was motivated by self-interest. He adds that colleagues inside Russia have not been silent: In September, hundreds of signatures were collected in an analogous appeal to the president by researchers inside the Russian Federation, including many prominent members of the Academy of Sciences.

—ANDREY ALLAKHVERDOV AND
VLADIMIR POKROVSKY

Andrey Allakhverdov and Vladimir Pokrovsky are writers in Moscow.

PLANETARY SCIENCE

Lunar Mission: A Slam, But Was It a Dunk?

NASA officials and scientists spent the better part of an hour at last week’s press conference on the Lunar Crater Observation and Sensing Satellite (LCROSS) mission patting themselves on the back. The mission was a success, they said, all the while ignoring a very large elephant in the room: No one among the millions watching as a 2-ton hunk of metal slammed into the moon could see the much-ballyhooed spray of dust and debris that they had been told to look for.

The impactor most certainly hit its target: the dark, frigid shadow inside Cabeus crater near the lunar south pole. And the nine instruments on the trailing LCROSS spacecraft returned all the planned data, which researchers will study for signs of water. But no visible flash was reported at the moment of impact, and no debris could be seen. “I’m not necessarily surprised,” said LCROSS principal investigator Anthony Colaprete of NASA’s Ames Research Center in Mountain View, California. In exploration, “you just never know how these things are going to go. We just have to go back with a finer-tooth comb.”

Actually, Colaprete had warned his colleagues, at least, about the possibility of a

no-show debris plume. “It’s a very unproven and highly unpredictable science, impact cratering,” he told an audience at the Lunar and Planetary Science Conference last March. Impact modelers working for the team had struggled to simulate the impact of a cylindrical—not a simpler spherical—



See anything? No dusty debris was seen after the impact, but hope remains for water vapor.

object, and one that was hollow, not solid, like the LCROSS impactor. Plus, it smashed into a surface of unknown shape and composition. LCROSS was “the most challenging impact modeling I’ve ever done,” said Erik

Asphaug of the University of California, Santa Cruz. There were just too many unknowns for him to be entirely comfortable with his results; impact on the odd unseen boulder, for example, could have sent most of the debris into the crater wall instead of into the sky.

LCROSS scientists may yet extract a debris plume from the data, but “the spectra is where the information is” about any water, Colaprete said, referring to spectral colors in the visible, infrared, and even ultraviolet returned by the trailing LCROSS spacecraft and by the Lunar Reconnaissance Orbiter. Some of these showed intriguing blips from the impact flash and the still-warm crater. There were also spectral changes above the impact site between pre- and post-impact. “What do these little blips mean? I don’t know,” Colaprete said. “I’m just glad they’re there. We’re going to work on this feverishly.” Determining whether it was water will take weeks or months of data combing, and no public word about water will be forthcoming before the December meeting of the American Geophysical Union, he said.

—RICHARD A. KERR



The Big Gamble in the Saudi Desert

King Abdullah University of Science and Technology is betting that generous funding and great facilities will attract the talent it will need to become a top-ranked institution

THUWAL, SAUDI ARABIA—The coral reefs that give the Red Sea its name provide a spectacular backdrop for the new King Abdullah University of Science and Technology (KAUST) here. The reefs also provide a unifying research theme for this lavishly endowed and precedent-shattering institution, which opened for business last month.

Scientists at the university's Red Sea Science and Engineering Research Center plan to use the reefs as the centerpiece for efforts to understand all aspects of their marine environment, from the genomic to the ecosystem level. They'll also be probing the interaction of the land, air, and water that shapes the entire region. Eight other research centers have equally broad mandates—from desalination and the genetics of plant stress to renewable energy and clean combustion—and several more are in the offing. Together, the centers embody KAUST's credo: To pursue cutting-edge interdisciplinary science on issues of global importance in areas where Saudi Arabia has a compelling national interest and, possibly, a comparative advantage.

"We want people with big ideas and big ambitions. Timid individuals need not

apply," says KAUST's president, Choon Fong Shih. A world expert in computational fracture mechanics, Shih helped lift the National University of Singapore into the top echelon of research universities, and last year he came to KAUST to achieve the same goal.

Thinking big is in the DNA of KAUST. The university's namesake, King Abdullah bin Abdulaziz Al-Saud, decided 3 years ago to set aside billions of dollars for an institution designed to help the country move from an oil-based to a knowledge economy (*Science*, 8 June 2007, p. 1409). The king chose Ali al-Naimi, the country's oil minister, to oversee

his vision, and Saudi Aramco, the giant state-owned oil and petrochemicals company and the country's most visible symbol of internationalism, to manage the project.

KAUST is perhaps the most-watched experiment in higher education taking place anywhere in the world. Its huge endowment is certainly an attention-getter: Two years ago, KAUST officials used a figure of \$10 billion, although one knowledgeable source says the number is actually \$20 billion. Saudi officials decided to make it a graduate-only university so that it wouldn't compete for undergraduates with existing Saudi institutions. KAUST hopes eventually to be the size of the California Institute of Technology—roughly 250 faculty and 2500 students—and to rival it in prestige. It's also the first Saudi institution of higher education to allow men and women to mix freely. But that policy has already been criticized sharply by conservative clerics from the country's dominant Wahhabi school of Islam and promises to be a source of continuing tension.

Academic oasis

The university sits on a 32-square-kilometer slab of desert an hour north of the port city of Jeddah, the jumping-off point for 4 million

Online

sciencemag.org

S Podcast interview with author Jeffrey Mervis.



Thirst for knowledge. A press conference during the inauguration of KAUST, which borders the Red Sea.

Muslim pilgrims headed to Mecca each year. Construction began barely 2 years ago, transforming this tiny fishing village into a teeming labor camp with as many as 30,000 workers. Last month, the 85-year-old monarch threw a party for some 3000 foreign dignitaries, visiting Nobelists, and the elite of Saudi society to showcase what his vast wealth has already created and, in a symposium on sustainability, to glimpse KAUST's future.

Classes began on 5 September for the initial class of 400 students, most of whom have signed up for master's degree programs. The founding faculty members have been trickling in over the past few months. Although it will be months before most are able to move into their own labs, professors already have access to core facilities that are second to none among research institutions. KAUST has already assembled what may well be the most ethnically and geographically diverse group of academics anywhere (see graphic, p. 356). On one measure of diversity, however, KAUST has a long way to go: There are only five women among the founding faculty, and all are junior (assistant) professors.

None of the new recruits ever imagined doing science in Saudi Arabia until they heard what KAUST had to offer. "I loved, really loved, working at Woods Hole [Oceanographic Institution]," says Michael Berumen, a 29-year-old assistant professor of marine sciences and the first hire by the Red Sea center's director, James Luyten, a former director of WHOI. "But field-based work on reefs is such a huge part of what I do. And now I have the ability to just pop on a boat for a couple of hours, to grab some coral samples or check on the fish we've just been talking about. And then you add in these incredible facilities."

To retain the talent being amassed, Saudi officials know they will need to maintain a high quality of life. So within the barbed wire and concrete barricades that encircle the campus, Aramco is building an entire community, with all the residential, retail, recreational, and cultural amenities that any academic scientist would expect. The thousands of palm

trees lining the main roads and academic complexes even help Berumen, an avid golfer, forget he's in a desert as he whacks balls at a driving range adjacent to a nine-hole golf course scheduled to open this month.

KAUST may be a generation or more away from distinguishing itself academically, but its management structure already sets it apart from every other research university. The biggest difference is the absence of academic departments. Instead, faculty are grouped into three interdisciplinary divisions that will, at the outset, offer degrees in nine fields. "It's the way you'd want to do it if you could start a university from scratch," says computational geophysicist Omar Ghattas of the University of Texas (UT), Austin, which has helped

KAUST recruit faculty and create its program in the computational sciences and engineering. "The traditional role of a university was to train people in the canon of each discipline. But today's biggest problems—energy, climate change, nutrition, water conservation—are interdisciplinary."

For most scientists, however, the biggest difference is how their research will be funded. Rather than having to submit a never-ending stream of grant applications to government agencies and face depressingly low success rates, each faculty member has been given substantial internal support—\$400,000 annually for assistant professors, \$600,000 for associate professors, and \$800,000 for full professors—from which

they can hire students and technicians, buy materials and supplies, travel, and otherwise tend to the needs of their individual labs. The funding supplements full access to core lab facilities that include a 220-teraflops IBM Blue Gene/P supercomputer that will be upgraded to a petaflops machine, an industrial-quality nanofabrication facility, a state-of-the-art visualization center, and 10 nuclear magnetic resonance machines, including a 950-MHz instrument that is not yet on the market. "It was like setting a bunch of kids loose in the best toy shop in the world," says Neil Alford, chair of the materials science department at Imperial College London (ICL), which helped KAUST assemble the noncomputing components of its core labs.

That's not all. Salaries are more than competitive with those in the West, say researchers, many of whom also receive free housing and other generous benefits. In fact, one prominent scientist wonders if the overall package may actually hinder KAUST's chances of producing great science. "I'm worried that they've gone beyond attracting people for the right reasons," says Northwestern University chemist J. Fraser Stoddart, a 2007 winner of the (Saudi) King Faisal Prize for his pioneering work in molecular recognition and self-assembly relating to nanosystems. "Money doesn't buy everything. After living such a palatial existence, people may find it difficult to leave."

At the same time, KAUST scientists will be working without an academic safety net. Instead of a shot at tenure, professors receive 5-year contracts that can be extended indefinitely so long as they remain productive. Nobody *Science* spoke to feels it's much of a sacrifice, however. "Tenure isn't a major factor for me," says Boon Ooi, a



Royal donation. King Abdullah, shown at the inaugural ceremonies, is funding KAUST, his namesake university.



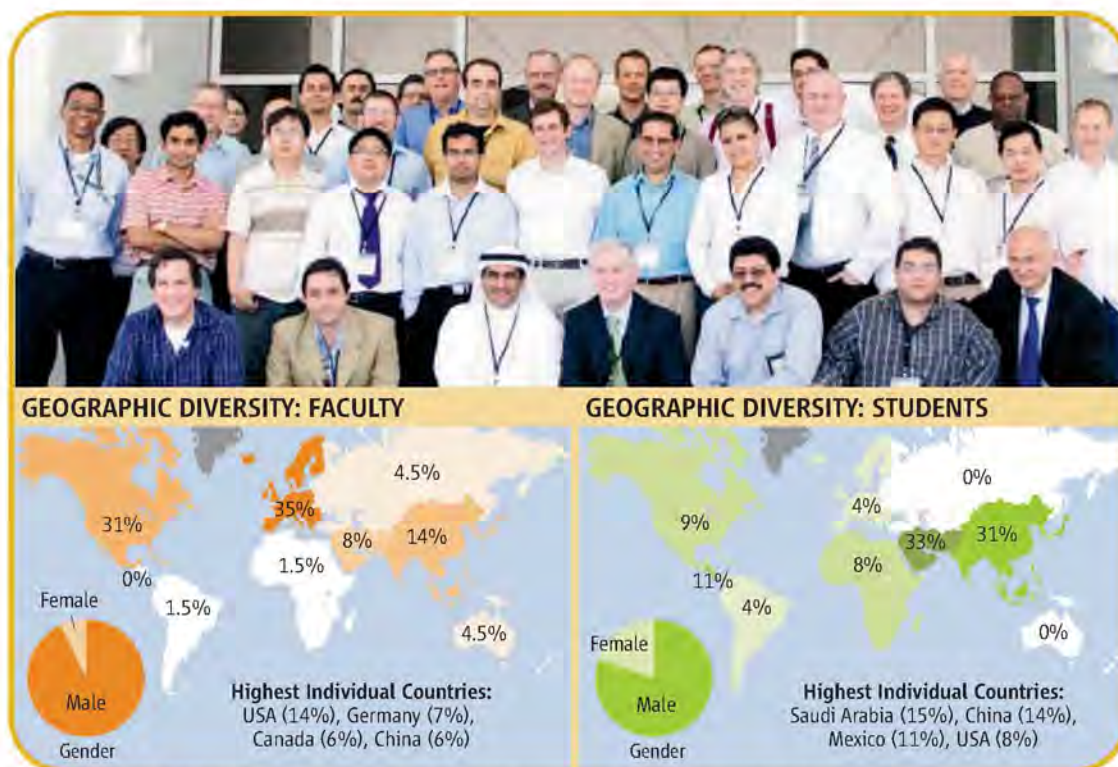
JIAN-KANG ZHU, DIRECTOR, PLANT STRESS GENOMICS AND TECHNOLOGY RESEARCH CENTER

Jian-Kang Zhu knows exactly what national leaders expect from his field of research, plant molecular genetics. The chairman of KAUST's board of trustees, also the country's oil minister, hopes that one day KAUST scientists will "find a way to grow wheat from ocean water." That's not likely to happen anytime soon, says Zhu, but the 42-year-old scientist says he relishes the challenge.

Born in China and trained in the United States, Zhu left a tenured position at the University of California, Riverside, this summer to pursue research on three groups of extremophiles—plants able to thrive amid drought, high salinity, and other extreme conditions—

that don't get a lot of respect from U.S. funding agencies because they are not crops. However, Zhu thinks they hold molecular secrets that can eventually be applied to increased food production in countries like Saudi Arabia, where such extreme conditions are the norm.

"I'm a scientist, and you go where you can do the best research. And right now that is at KAUST."



Global outlook. KAUST's founding faculty and students come from around the world, but most are male.

Malaysian-born professor of electrical engineering who had just been awarded tenure at Lehigh University in Bethlehem, Pennsylvania, when he decided this spring to come to KAUST to work on quantum nanostructures and their use as optical sensors. "I think it only slows down the pace of your research." Ooi is certainly not afraid of change. His academic odyssey has taken him from Glasgow to Singapore to the United States, with a 3-year break to create, run, and then sell off a photonics company.

Even before Ooi and others arrived, they had begun recruiting the graduate students (meaning those already holding master's degrees) and postdocs needed to make their labs productive. KAUST effectively bought its initial group of students by paying for the students' last two undergraduate years in return for a nonbinding promise to apply to its master's programs. Most have kept their word.

KAUST has also coaxed up to the best academic programs and scientists in the world. One innovative approach, called the Academic Excellence Alliance, paid a handful of departments (including UT Austin in computational science and ICL in materials science) \$25 million each over 5 years to identify and vet the best scientists in their field from around the world, many of whom wound up being hired. The alliance partners have also contributed curricula and begun research collaborations.

Corporate culture

Although Shih has promised that KAUST "will play by the rules" in terms of the free and open exchange of ideas and data, routine financial information about the university itself is hard to come by. Asked if a \$3 billion figure in the local press for the cost of construction was accurate, Shih would say only that "we are grateful to Aramco for paying the full cost of developing, constructing, and outfitting KAUST's physical infrastructure and laboratories." KAUST's operating



DAVID KEYES, DEAN, MATHEMATICAL AND COMPUTER SCIENCES AND ENGINEERING

Douglas Arnold, president of the Society for Industrial and Applied Mathematics, says he was a bit surprised when he heard that his colleague—and SIAM's vice president—had decided to join KAUST. "Isn't that a bit much?" Arnold recalls asking David Keyes. But Keyes's answer—"It's a great adventure, and a unique opportunity"—was no surprise to Arnold.

"David is a powerhouse and a visionary, someone who's extremely energetic—and a bit overextended," explains Arnold, a professor at the University of Minnesota, Twin Cities. "He cares a great deal about the role of computing in science and engineering, and he has a great sense of duty and service. He's also used to doing a lot of flying."

Keyes, 52, is officially on leave from Columbia University. "I never really leave a place," he confesses, noting that he has juggled academic appointments at Yale, Old Dominion, and Columbia universities with long stints at NASA and Department of Energy labs. But he says he plans to work full-time to help KAUST achieve its potential: "I can't think of a better place to advance computational science and engineering." His wife, a violist, hopes to do her part by developing a cultural arts program on campus.

budget, generated from the endowment and believed to be between \$700 million and \$1 billion, is also a secret, as is the size of the endowment itself. "I don't even know," says Nadhmi Al-Nasr, interim executive vice president of administration and finance. But he adds that "we were not impacted by the economic downturn" because investment managers "didn't even decide to enter the market until the whole thing was over."

The dominant role of Aramco in the university's affairs also chafes. Aramco not only provides financial support, but many senior administrators, including Al-Nasr, are on loan from the company. Several faculty members complained to *Science* about certain corporate policies, from prescribed work hours to procurement practices, that

they say are inappropriate for an academic setting. Some wonder whether Aramco's power over the university's purse strings will also give it undue influence in shaping academic policies.

The sudden departure this spring of its founding provost, a prominent academic with deep roots in the region, reflects some underlying tensions within the top university leadership on managing that relationship. On 30 March, the KAUST faculty members were stunned to learn via an e-mail from

Shih that Fawwaz Ulaby, a pioneer in radar remote sensing and a member of the U.S. National Academy of Engineering, was stepping down after only 1 year on the job. A charismatic promoter of the nascent university and an indefatigable traveler, Ulaby had recruited most of the faculty members. And his heritage—born in Syria, he was educated in Lebanon before coming to the United States for graduate study—was a source of great pride to Saudi officials.

Rumors were rampant that Ulaby had suffered a heart attack during an intercontinental flight and that his heavy workload was ruining his health. Shih's short note said only that Ulaby had "considered the needs of his family" before deciding to step down, and Shih added last month that Ulaby's decision to leave "was entirely personal." But Ulaby, now back at the University of Michigan, Ann Arbor, says the real reasons are entirely professional, not personal. "I categorically reject [the suggestion] that I stepped down for health reasons," the 66-year-old electrical engineer tells *Science*. "I am as fit as can be."

Ulaby says he quit "because I was no longer allowed to do my job as provost." That job included overseeing a seamless integration of the university's degree programs, research centers, and extensive core facilities. Instead, he says, the enormous challenge of building a graduate university from scratch and the imperative of opening on time led to "impossible disconnects" such as "hiring faculty and graduate students in chemistry when there is no degree program in chemistry. That happened, and it doesn't make any sense." Ulaby says his attempts to "integrate those elements in an amicable fashion" were thwarted.

Ulaby also worries that a corporate mentality is being applied to an academic setting. "KAUST has not created an environment in which faculty can enjoy a significant degree of freedom in carrying out their research," he says, citing as one example the high-level, prior approval needed for travel to conferences and visits to other institutions. Ulaby says he still believes in the university's strategic goals—"to be small but spectacular in quality and scientific impact"—but that current policies may make them harder to achieve.

Waiting to start

The biggest complaint among faculty members is that they have been unable to start their research on campus. Although they are teaching classes, faculty members expect it to be another 6 to 12 months before they can move into their own labs. "This project is huge, and

there are so many details," says Kenneth Minneman, dean of chemical and life sciences and engineering and a former long-time professor of pharmacology at Emory University in Atlanta. "Somehow they lost sight of the fact that labs are an essential element of a research university. But the people at the top are now aware of the problems, and they know what needs to be done."

Mohamed Samaha, interim senior vice president for research and economic development, explains that getting the core facilities up and running was his first priority. "The next order of business is giving faculty



NIVEEN KHASHAB, ASSISTANT PROFESSOR, CHEMICAL SCIENCE

When Niveen Khashab called with the news that she had accepted a position at KAUST, her mother fainted. Then she couldn't stop crying. Her dream had come true. Her daughter was coming home.

Khashab, 27, had grown up in Lebanon to parents of Saudi and Lebanese heritage and did her undergraduate work at American University of Beirut. In 2002, she left for the United States to earn a Ph.D. in chemistry at the University of Florida, Gainesville. After doing a postdoc with nanotechnology pioneer J. Fraser Stoddart, she figured her next step would be a tenure-track position at a major U.S. research university.

"My mother always wanted me to come back to the Middle East," says Khashab, whose Lebanese-born husband works in KAUST's IT department; their 3-year-old daughter attends the Western-style preschool on campus. "But I kept telling her that it would never happen. There was no place in the region where I could do what I wanted to do." Until KAUST, that is.

the attention they need," he says. Swing labs that will provide temporary space are scheduled to open as early as next month.

The delay is a minor impediment for computational scientists and engineers, says David Keyes, dean of mathematical and computer sciences and engineering. "We're internationally portable," he quips. "But those who have to get into a system or develop a process are feeling the delays more acutely." That would include Niveen Khashab, an assistant professor of chemical science who is using silica-based nanomachines to develop better drug-delivery systems. A former postdoc in Stoddart's lab, Khashab is coping by voluntarily teaching a second class this semester, twice the normal workload. In return, she's hoping next spring to work full-time in her lab.

Khashab is one of only five women among the founding faculty. Shih says that's a concern and adds that he plans "to redouble our efforts to recruit women." But he isn't sanguine about his chances of success. "There is a shortage of women everywhere, especially at the senior level, and we are competing with the rest of the world," he

says. About 20% of the students in the entering class are women, a percentage comparable to many U.S. graduate programs in the physical sciences and engineering.

University officials hope that KAUST's presence will cause Saudi universities to raise their game. More grandly, Saudi officials talk about KAUST making the Middle East a major player in science and technology for the first time since the golden age of Islamic culture a millennium ago.

Michel Bercovier, a prominent computational scientist at The Hebrew University of Jerusalem, thinks that could happen and says

he's been impressed with the caliber of talent from his field that KAUST has already attracted. "KAUST is more than interesting, it's important. And politicians need to be aware of it," he says. "I hope that KAUST will be a Sputnik moment for science in the Middle East."

Ironically, politics may also prevent Bercovier and other Israeli scientists from participating. Bercovier says he will measure KAUST's success by its ability to host top scientists from around the world for workshops and conferences. But because Saudi Arabia has no diplomatic ties with Israel, and the average Israeli citizen cannot obtain a visa to enter the country, his country's scientists won't be participating in KAUST-sponsored activities anytime soon.

Given the kingdom's hard line on Israel and the large role of religion in Saudi society, Shih acknowledges that KAUST's ability to crack the top 20 list of research universities will depend in part on the Saudi government. "We are an academic institution, and we have to work within the laws of the country," he says. "There are much larger issues here."

—JEFFREY MERVIS

Fetal Cells Again?

Despite past failures and growing skepticism about cell therapy in general, scientists once again plan to test fetal cell transplants on Parkinson's disease

In 1987, an American neurologist told *The New York Times*, "I think I witnessed history," after watching Mexican surgeons perform a transplant of human adrenal gland cells into the brains of four people with Parkinson's disease. The physicians anticipated that the cells would turn into producers of the neurotransmitter dopamine, replacing the striatal neurons whose degeneration was clearly at the heart of the condition. At about the same time, Swedish doctors were experimenting with transplanting cells from fetal brains, a controversial strategy that produced such striking results in some cases that by 1997 about 200 patients around the world had received the treatment.

But two fetal tissue trials in the United States in the 1990s that used sham surgeries as controls burst the balloon, indicating that any benefits may be little more than a placebo effect. Moreover, many of the patients developed "dyskinesias"—uncontrolled movements from excess dopamine from the brain grafts. It was a huge step backward; cell therapy for Parkinson's was all but abandoned.

Then this decade, human embryonic stem (hES) cells came onto the scene, and scientists began trying to convert these malleable cells into dopamine-producing neurons that might provide a safer, more abundant and less controversial source of transplantable tissue than aborted fetuses. More recently, induced pluripotent stem (iPS) cells have been added to the mix. But as the lab research proceeds apace, there's growing doubt in some quarters about whether cell transplants will ever show a clear benefit for Parkinson's disease beyond what can be achieved by existing therapies. And researchers are increasingly realizing that there's much more to Parkinson's than a dopamine shortage.

The debate about cell therapy for Parkinson's may soon become more intense as scientists in Europe, in collaboration with colleagues in North America, are in final negotiations for a large European Commission grant to conduct a

new fetal cell transplant trial. The trial will refine clinical methods and be a "stepping-stone" to therapies with cells derived from iPS or hES cells, says the principal investigator, neurologist Roger Barker of the University of Cambridge in the United Kingdom. Yet news of



Taking the plunge. Surgeon Ivar Mendez readies a needle containing fetal brain cells.

the plans dismays some. "I think it's a step backwards; ... all the double-blind trials have failed," says C. Warren Olanow, a neurologist at Mount Sinai School of Medicine in New York City, who headed one of those trials.

Going head to head

A half-dozen years ago, in the heat of political and scientific excitement over hES cells, Parkinson's disease was regarded as one of the prime candidates for stem cell therapy. But even as iPS cells have opened new vistas, the prospect of cell therapy trials has been steadily receding as scientists have gained new appreciation of both the difficulties of cell culture and the complexity of the disease itself.

All over the world, researchers are trying to develop dopamine-producing cells from animal and human ES cells and from iPS cells.

Although many have generated human dopamine-producing neurons in a dish, no one has proven that they have exactly the right kind: differentiated enough so the cells will not develop tumors but young enough to grow into the proper type of cell and make connections.

So far, says Lorenz Studer of Memorial Sloan-Kettering Cancer Center in New York City, researchers have gotten things right with mouse cells. A big push now is testing human-derived cells in primates to find out what type is most likely to survive and build connections within the brain without forming tumors. For example, Evan Snyder of the Burnham Institute for Medical Research in San Diego, California, and colleagues are testing a half-dozen human cell types—including fetal brain cells, adult neural stem cells from cadavers, and ES and iPS cells at various stages of development—to see if they will engraft successfully in monkeys. "Nobody's ever compared them head to head," says Snyder, who wants to ascertain the optimal stage of differentiation, the best place in the brain to insert cells, and whether the transplanted tissue needs to just churn out dopamine or reconstruct the whole striatal pathway, the neural circuitry that degenerates in Parkinson's.

Studer points out that criteria have become very demanding for any such cell treatment: "To have a clinical trial, you have to have a treatment that promises to be better than anything now available." Deep-brain stimulation, adopted in the 1990s, has raised the bar considerably: It can help patients when L-dopa medication loses its impact and can curb dyskinesias from long-term exposure to the drug (*Science*, 20 March, p. 1554). Studer predicts that in optimal circumstances—following no-glitch studies treating Parkinsonian monkeys with human dopamine-producing cells—it would be at least 5 years before any clinical trial.

Back to fetal tissue

As these challenges have become clearer, some scientists have spent the past 3 years discussing a way to push the field ahead while human ES and iPS culture techniques are being perfected: a return to fetal tissue transplants. Despite the failure of the U.S. trials, which were funded by the National Institutes of Health (NIH), two uncontrolled trials held in Sweden in the 1990s appeared to produce striking improvements—with a handful of patients doing well up to 14 years later.

These results have convinced some scientists that it's time to try again. If all goes as planned for the European Commission-sponsored trial, the first patients in a preliminary safety trial will receive injections of cells from the midbrains of 6-to-9-week-old fetuses in 2012. After that, a double-blind treatment

trial—which Barker says would include sham surgeries “when we feel we have an optimal therapy for grafting”—will recruit patients at several centers in Europe and North America.

Although Parkinson’s disease researchers in Europe are generally enthusiastic about the plan, reaction among U.S. scientists is mixed. Ted Dawson, professor of neurodegenerative diseases at Johns Hopkins University in Baltimore, Maryland, finds it “surprising. ... I had thought there was going to be a moratorium [on fetal tissue transplants] until we had a better understanding of dopamine neurons in transplantation.” Some scientists also point to long-term follow-up results reported last year in *Nature Medicine*, which revealed that the disease process had begun to affect a small portion of the fetal grafts in some patients.

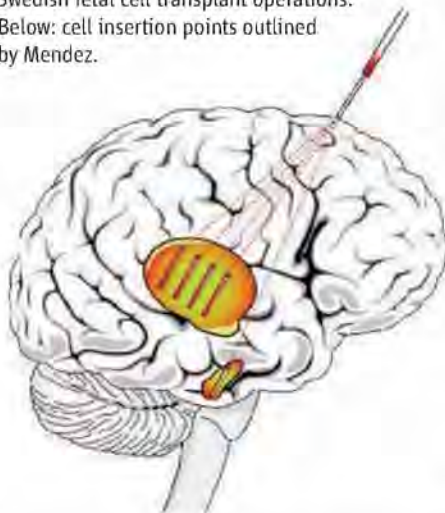
But psychiatrist D. Eugene Redmond of Yale University, who has done fetal tissue transplants in a monkey model of Parkinson’s, points out that if you want to do a test of cell replacement, fetal brain tissue is currently “pretty much the gold standard.” Those prenatal dopamine neurons are at just the right stage of development and don’t cause tumors, he explains. And they work better in monkeys (where scientists use a chemical to selectively kill off striatal dopamine cells) than do dopamine-producing cells grown from monkey ES cells, Redmond says.

The European group insists that the new trials will be better controlled in every way than were those held in the 1990s. “We think the NIH trials were held at a time when several aspects of the technique were not fully developed,” says Anders Björklund, a fetal transplant pioneer at Lund University in Sweden. Barker says the team has identified some of the “critical factors” responsible for the failures. One is the mix of cells in the fetal tissues that were transplanted. Recent animal work in Björklund’s lab has shown that if the fetal brain tissue contains a high proportion of dopamine neurons, they will offset possible dyskinesia-causing effects from other types of cells in the graft, principally serotonin neurons. To get the right mix, says Barker, there will be more “selective dissection” from the fetal brains (four to six are needed to treat each patient). Patients will also be younger (under 60), and with the aid of brain imaging, researchers will pick those whose dopamine loss is more restricted to the dorsal striatum.

Neurosurgeon Ivar Mendez of Dalhousie University in Halifax, Canada, who has already reported success with implanting Parkinson’s patients with fetal dopamine neurons, has worked out a standardized surgical procedure with the “Halifax injector,” a computer-controlled system for adminis-



Old and new. Above: one of the first Swedish fetal cell transplant operations. Below: cell insertion points outlined by Mendez.



tering precise amounts of cells at specific brain locations through a hole in the skull. Mendez says about 4 million fetal brain cells—equivalent to about a drop of tissue—will be injected deep in each side of the brain. The placement of the graft deposits will cover as much of the dopamine-depleted striatum as possible, says Björklund.

Neurologist Olle Lindvall of Lund University says the new trial will be able to address the “big question”: Why does the treatment bring “major recovery ... in some patients and not in others”? Barker says that the answer may come from new information revealing subtypes of Parkinson’s. Barker readily admits that cell therapy “is not a cure” but contends that it’s an “open question” whether cell grafts are superior to deep-brain stimulation.

Some U.S. observers agree that the trials are worth doing. “There’s a lot of [unwarranted] bias in the field because of two [U.S.] clinical trials,” says Ole Isacson of Harvard Medical School in Boston. Redmond agrees: “A whole lot more is known about fetal tissue transplants than was known [in the 1990s]. It makes sense to continue looking.”

The rest of the iceberg

To others, the return of fetal cell therapy fails to give enough heed to recent advances in the

understanding of Parkinson’s disease. Stem cell treatment “looked most hopeful when people were treating [Parkinson’s] just as a dopamine disease,” says Olanow. Degeneration of dopamine-producing cells is not the first or the only symptom of Parkinson’s, however. It’s become increasingly clear that, as neurologist J. William Langston of the Parkinson’s Institute and Clinical Center in Sunnyvale, California, has put it, “Parkinsonism [that is, dopamine-related movement problems] is just the tip of the iceberg.” In reality, says Olanow, there is “very extensive pathology” that covers many neurotransmitter systems as well as the autonomic nervous system. Non-dopamine symptoms include bowel and bladder problems, attacks of low blood pressure, falling, “freezing,” sleep disorders, pain, depression, speech difficulties, and dementia.

“If patients’ only problems were related to dopamine deficiency, we would be able to maintain [them] for decades” with drugs and deep-brain stimulation, says neurologist J. Eric Ahlskog of the Mayo Clinic in Rochester, Minnesota. But given the extensive nature of the disease, any cell therapy “would need to be broadly administered and not just in one of a few brain regions.” It may be that cell transplants ultimately are most useful in furnishing trophic factors, protective chemicals that stave off deterioration of existing neurons—as some researchers hope will work in the case of amyotrophic lateral sclerosis.

Olanow notes the limitations that pertain to dopamine-focused cell therapy also apply to current Parkinson’s disease gene-therapy efforts, which center on introducing one or more genes involved in dopamine synthesis. Two groups, including one reporting in the 14 October online issue of *Science Translational Medicine*, have evidence that such gene therapy can restore dopamine production without associated dyskinesias in monkeys. This strategy has now moved into clinical testing. It’s “exciting work,” says Olanow, but he contends that “the near-term future of cell and gene therapies based on dopamine restoration don’t look particularly promising.”

The Michael J. Fox Foundation for Parkinson’s Research has also become much more cautious about the promise of cell therapy. The foundation is now placing its bets on new drug development and supports very little stem cell research. “I was totally naïve when I came to the foundation” in 2002, says CEO Katie Hood. “All my exposure was pop media; I thought it was all about stem cells.” Now, she says, “I have not totally lost hope on cell replacement,” but “I just don’t think it’s a near-term hope.”

—CONSTANCE HOLDEN



NEWSMAKER INTERVIEW: M. S. SWAMINATHAN

A Guru of the Green Revolution Reflects on Borlaug's Legacy

NEW DELHI—During the Green Revolution of the 1960s and 1970s, Norman Borlaug's innovative semidwarf, rust-resistant wheat varieties, honed in Mexico, produced bumper crops—and saved countless lives (see p. 381). Average wheat yields in Punjab, for example, are 4.5 tons per hectare—more than five times higher than in pre-revolutionary times. Borlaug's standard-bearer in India was agricultural scientist Monkombu Sambasivan Swaminathan, now a parliamentarian and chair of the M.S. Swaminathan Research Foundation in Chennai. Swaminathan, 84, worked with Borlaug for nearly half a century and spoke with *Science* about the Nobel laureate's contributions to South Asia.

—PALLAVA BAGLA

Q: What was Dr. Borlaug's impact in India?

M.S.S.: Borlaug was the catalyst of change, a catalyst in the sense he brought these new varieties, these seeds of change.

Q: Is there any estimate of how much of the wheat grown in India has signatures of the original material from Mexico?

M.S.S.: Almost 100% [have at least] a few genes of the original material, particularly the dwarfing gene, and maybe there are a few genes for resistance to pests and diseases.

Q: So Borlaug still lives and breathes in Indian farmers?

M.S.S.: He lives through the genes.

Q: Was Borlaug's contribution merely science, or did he go beyond science as well?

M.S.S.: The Green Revolution was a synthesis of three elements: science, which is a prime mover of change, because we didn't have the new type of plant. Then public policy, and above all, farmers' enthusiasm. When we started the program, people discouraged us, saying farmers will not take your variety because it is short—lacking enough straw to feed livestock. We proved them all wrong, all those prophets of doom.

Borlaug brought a revolution in ideas, a revolution in thinking, a revolution in technology. It's a totality. It was a great social change. It's quite likely that a billion people have been saved in India, Pakistan, and Bangladesh.

Q: Did he worry that there are so many who are still malnourished and hungry?

M.S.S.: He was a man of the field. He didn't like sitting in a room, in a laboratory; he was not very comfortable with seminars

Retrospective on
Norman Borlaug.
Page 381



Comrade in arms. Borlaug (above) lives on in India through the genes of his Mexican wheat varieties, says Swaminathan.

and symposia. He got impatient with ministers who are dining and wining and eternally talking poor and living rich.

Q: But why are so many people still so malnourished?

M.S.S.: Because of lack of money, lack of purchasing power. Indian famines are not famines of food anymore.

I am happy in some respects. For example, our average life span has gone up enormously. What I am not happy about is that India still is home for the largest number of malnourished children, women, and men. I think it is inexcusable. It's very sad: A country which can make a nuclear submarine is allowing grains to be eaten by rats.

Q: Did Borlaug reflect on this?

M.S.S.: He was very perturbed by what is happening in India. As a humanist, he was deeply concerned, particularly about the issue of farmer suicides and why should it happen in a country like India—a democratic nation which ought to attend to the problems of its people.

PROFILE: VEERABHADRAN RAMANATHAN

From Burning Dung To Global Warming And Back Again

His childhood in rural India inspired the latest twist in climate scientist V. Ramanathan's long career studying—and now fighting—climate change

SAN DIEGO, CALIFORNIA—From his corner office on the terraced seaside campus of the Scripps Institution of Oceanography, Veerabhadran Ramanathan can look out 25 kilometers across the blue Pacific Ocean on a clear day. When San Diego's pollutant "brown cloud" blows in, the dim view reminds him of his current scientific bread and butter: the pernicious boost that such hazes give to global warming.

More personally, the brown smudge on the horizon takes him back to his childhood summers in rural southern India half a century ago, where his grandmother would cough endlessly over her smoky indoor cooking fire of sticks and dung. Fires like hers still stoke the mother of all brown clouds, the one over South Asia.

That connection helps explain Ramanathan's latest zigzag in a career full of unpredictable redirections. After discovering the unrecognized warming threat of trace greenhouse gases, provoking a reexamination of tropical meteorology, and revealing the insidious climate effects of brown clouds, the 64-year-old climate scientist is now going back to rural India. There he hopes to show how today's rural Indian women can cook more cleanly than his grandmother did while staving off disastrous global warming.

Whether discovering a new global warming threat or testing a new cooking stove, Ramanathan "really is bold," says Ralph

Cicerone, president of the National Academy of Sciences and a 35-year colleague of Ramanathan's. "Though he's very mild-mannered, there's an internal drive that's pretty fierce."

An aimless beginning

That drive came late. From his years working on his bachelor's degree in engineering at the Annamalai University in Chidambaram, south India, Ramanathan says, "all I can remember is honing my skills in tennis and table tennis. I had this vision of being a tennis star." Academically, "I had no goals for myself," he recalls. He did bring a certain independence of mind to his studies. When Ramanathan—Ram for short—was 11, his father, a traveling salesman for Goodyear Tire and Rubber Company, moved the family from Madurai to Bangalore. School there was taught in English, not Ramanathan's native Tamil. While picking up English, "I lost the habit of listening to teachers" he couldn't understand, Ramanathan says. "I had to figure out everything on my own. It helped me enormously in research."

After graduating from the university in 1965, he took a job at a refrigerator manufacturing plant. "Two years into it, I hated it. My job was preventing the [refrigerant] chlorofluorocarbons from escaping; I was not successful." He quit manufacturing and went back to school for a master's degree in engineering. There he got his first taste of research: building India's first Mach-Zehnder interferometer, an optical instrument for studying turbulent fluids. "I hadn't felt capable of anything like that," he recalls. "That gave me confidence."

Off to the planets

Research was not popular in India, however, and Ramanathan was reluctant to go back to manufacturing. "My dream was to come to America and drive American cars and enjoy

the good life," he says. So he wrote to fellow engineer Robert Cess of the State University of New York at Stony Brook (now Stony Brook University) asking about graduate work with the university's brand-new Mach-Zehnder interferometer. Cess took him on but "got bored with what I was doing" just as Ramanathan arrived, says Cess. He switched from studying combustion to studying the planets, taking Ramanathan with him. They applied an engineer's understanding of radiative transfer—the way heat is emitted, absorbed, and scattered—to the nature of the atmospheres of Venus and Mars and the way carbon dioxide traps radiation to produce a greenhouse. That was when "I realized I'd found my calling," says Ramanathan, "working on the natural environment."

No climate jobs came up, but Ramanathan's radiative-transfer expertise won him a postdoctoral position in a NASA laboratory that applied radiative transfer to the problem of how spacecraft can blaze safely home through the atmosphere. Then his new boss, like Cess, switched fields, putting him to work on how ozone in the stratosphere influences surface climate.

This latest random twist in the road carried Ramanathan into climate for good. At an ozone workshop, he learned of a recent landmark paper that tied chlorofluorocarbons (CFCs) to the chemical destruction of stratospheric ozone. Ramanathan recalled from his refrigerator days that CFCs would trap heat escaping from Earth and add to greenhouse warming. But were CFCs powerful enough greenhouse



Ticket to ride. Building an interferometer took Ramanathan to the United States.

CREDITS (TOP TO BOTTOM): H. V. NGUYEN, C4/SIO/UCSD; COURTESY OF VEERABHADRAN RAMANATHAN

Something in the air. This brown cloud—fueled by burning fuels in India—led Ramanathan to fly instrumented drones through polluted clouds.

gases to compensate for their parts-per-trillion abundance in the atmosphere?

Working nights, “I did the calculation six times,” he says, and every time the radiative transfer calculation showed that each CFC molecule was 10,000 times more effective as a greenhouse gas than was carbon dioxide. The result became the crux of his first single-author paper, a blockbuster in *Science* in 1975 that launched an entire subfield of climate research. Eventually, Ramanathan and others found that rising trace gases such as CFCs account for 45% of the drive behind greenhouse warming from gases.

Against the tide

Provocation comes naturally to Ramanathan, says his wife of 36 years, Giri Ramanathan. “What Ram is good at is being original,” she says. “He loves going against the tide, he loves to get people on his bandwagon.” After a stint at the National Center for Atmospheric Research in Boulder, Colorado, where he helped build NCAR’s first world-class global climate model, Ramanathan moved on to the University of Chicago in Illinois. There he and his postdoc proposed a provocative hypothesis: Increasing clouds intervene to limit the greenhouse warming due to water vapor. In 1993, Ramanathan co-led his first major field study, the \$20 million Central Equatorial Pacific Experiment (CEPEX), drawing on ship, plane, satellite, and balloon observations to test this “thermostat hypothesis.”

Tropical meteorologists objected vociferously. “I didn’t handle the controversy right,” says Ramanathan. At a meeting, “I pounded the table; I said something that made the community angry. I let personality come in the way.”

Most researchers have since concluded Ramanathan—who has withdrawn from that field—was largely mistaken though perhaps ultimately constructive. “I think his [thermostat] paper is one of the most important in the meteorology of the tropics,” says tropical meteorologist Peter Webster of the Georgia Institute of Technology in Atlanta. “Not because it’s right—I think it’s a little wrong—but because it acted as a catalyst to get people thinking. It shows what a strong, ambitious scientist can bring about.”

To the brown cloud’s heart

CEPEX may not have won the day for Ramanathan, but it did point him to the remainder of his life’s work. CEPEX observations suggested to him that climate models

were doing a lousy job of simulating the effect of aerosols, the microscopic particles of dust, sea salt, and pollutant crud that form a sun-dimming visible haze. To find out, Ramanathan co-led with Nobelist Paul Crutzen the \$20 million Indian Ocean Experiment (INDOEX) in 1995 involving six aircraft and 200 international scientists.

INDOEX was wildly successful, unfortunately. Researchers flew into an awe-inspiring brown cloud 3 kilometers thick spread over an area the size of the continental United States. It was so dense that it reduced sunlight reaching the surface by as much as 10% to 15%, an effect missing in the models. The problem was soot. The brown cloud’s particles incorporated black carbon spewed by combustion—burning coal, diesel engines, and dung fires like the one Ramanathan’s grandmother used to cook on. Black carbon-laden aerosols absorb sunlight and warm the air, boosting global warming. They may even be suppressing monsoon

good.” At about the same time, he was shaken by the new science about the brown cloud over Asia. He learned that “most of the black carbon is from biofuel burning,” he says. “That was it. It took me back to what I had seen in my childhood” watching his grandmother coughing over her cooking fire.

Then, 3 years ago, he got yet another push. At the United Nations, “I gave a passionate speech” about global warming to an international group of high school students. “A shy African girl asked, ‘What are you personally doing about this problem?’ I had nothing to say.”

On a personal level, he started taking the bus from home to Scripps and installed solar-electric panels on his house. More globally, he has launched Project Surya—Sanskrit for “sun.” Surya “was a gift from God ... that I have a chance to go back and fix an age-old problem.” Surya is an experiment aimed at someday clearing a major part of South Asia’s



Looking for a fix. Ramanathan is looking to clear the air with cleaner cook stoves in northern India.

rainfall, depressing Indian agricultural production, and melting Himalayan glaciers, as Ramanathan has argued.

Getting personal

INDOEX was a turning point. On the last INDOEX flight, into the Bay of Bengal off southern India, “I saw a vast cloud,” Ramanathan recalls. “I grew up in southern India. I thought, ‘I can’t leave these millions of people to deal with this on their own.’ I knew this is where I was going to spend the rest of my scientific career.”

That commitment evolved when Ramanathan turned 60 in 2004. Crossing that threshold “makes you look back,” he says. “I’d been working on [climate change] 35 years. All I had done was produce one bad-news paper after another. I had to do something

pall of brown clouds through cleaner ways of cooking. Ramanathan plans to put cleaner-burning cook stoves and solar stoves into the hands of those living in two rural areas of about 50,000 people each in the north of India and monitor the effects. Ramanathan expects to see dramatic declines in airborne black carbon both in their homes and in and near the villages.

Fundraising has been slow so far. Ramanathan has put in \$15,000 of his own money (he recently shared the \$200,000 Tyler Prize for Environmental Achievement) and raised more from colleagues and foundations, although a large grant still eludes him. But there’s no stopping, he says. “If you accept it’s a problem, then you have to do something about it.” If the 4 billion people using biofuels “go the fossil fuels route, there is no hope.”

—RICHARD A. KERR

To check *Plasmodium*

369



How tyrannosaurids evolved

373



LETTERS | BOOKS | POLICY FORUM | EDUCATION FORUM | PERSPECTIVES

LETTERS

edited by Jennifer Sills

Turning Ivory Towers into a Golden Economy

IN EARLY MAY, STEVEN CHU—NOBEL PRIZE WINNER AND THE SECRETARY OF THE U.S. Department of Energy—announced that the federal government would underwrite the formation of eight energy research hubs. Somewhat modeled on Bell Labs, the energy hubs would help scientists take basic research discoveries and translate them into commercially viable technologies. This is a good first step toward better capturing the vast commercial potential residing in America's research laboratories and inside the minds of our talented graduate student researchers and scientists. Unfortunately, scientists and graduate students lack entrepreneurial

skills, and few universities have aggressive plans to go beyond research to the translation of research into practice. Something is missing from teaching curricula, research plans, and federal funding policies.

We propose that translational research education be offered to every one of the 30,000 Ph.D.s graduating each year in science and engineering fields. Each student would have the opportunity to perform an impact or market analysis of his or her field; take mini-courses tailored to Ph.D.s in subjects such as business skills,



The scientist entrepreneur's toolbox.

finance and accounting, science policy, and entrepreneurship; and receive mentoring from successful entrepreneurs and from faculty from outside the sciences on how his or her work is informed by and affects society at large. If one in every five to ten Ph.D. students were to take on this extra dimension to their training, and if startup resources were provided for the top 20%, the total cost would be on the order of 1% of the federal basic research budget.

With little upfront investment, we could turn universities from basic science discovery institutes into powerful science and technology innovation engines. In as little as 2 years, such a program could have a strong positive impact on the economy.

Of course, not all scientists should become entrepreneurs. But more than ever before, students in the sciences are ready to follow the passionate path of entrepreneurship. Recent surveys from the National Academy of Engineering (1) have found that, more than at any time in recent memory, young engineering students want to use their education to improve the world. This is the classic definition of an entrepreneur.

Federal investment is needed because unlike undergraduate and master's education, doctoral

education is funded almost entirely through faculty research projects. There is no agency with a mandate to accept proposals for translational research and education except on a pilot level—but we need our government to take ownership of this problem and the exciting possibilities a solution holds for our country.

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Bushmeat Hunting As Climate Threat

TROPICAL FORESTS STORE 340 BILLION TONS of carbon, equivalent to more than 40 years' worth of human fossil fuel emissions (1). Tropical deforestation and degradation are responsible for an estimated 20% of global carbon emissions to the atmosphere (2). In response, a new policy that aims to reduce emissions from deforestation and degradation (REDD) will likely be part of the climate treaty negotiated by the United Nations Framework Convention on Climate Change this December.

Meanwhile, overhunting is pushing many animals to extinction (3, 4). Hunting rates in tropical Africa are more than six times greater than sustainable levels; large animals are already gone from most tropical Asian forests (4). Losses from overhunting are particularly severe among large-bodied animals because these species tend to be preferentially hunted and to have slower population growth rates (5).

This "bushmeat crisis" has received growing attention from vertebrate ecologists but is rarely considered by climate change scientists. Yet the two issues are linked: By removing the animal dispersers of carbon-rich tree seeds, hunting may be reducing the globally impor-

Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the previous 3 months or issues of general interest. They can be submitted through the Web (www.submit2science.org) or by regular mail (1200 New York Ave., NW, Washington, DC 20005, USA). Letters are not acknowledged upon receipt, nor are authors generally consulted before publication. Whether published in full or in part, letters are subject to editing for clarity and space.



tant carbon sink provided by tropical forests. Many of the most carbon-dense tree species rely on large vertebrates to transport their seeds and ensure successful reproduction.

Recent work in hunted forests of Peru reveals a substantial shift in species composition as large-seeded trees are replaced by smaller-seeded species (6). Furthermore, several studies highlight a positive relationship between seed size and tree wood density (7, 8). Wood density is among the best predictors of aboveground carbon storage in tropical forests (9, 10). Simulations of species composition in Panama show that selective logging of trees with high wood density reduces forest carbon storage by a staggering 70% (10). We suggest that degradations of carbon storage can also be driven by overhunting, as large-seeded trees with high wood density are deprived of their seed-dispersing animals. Even forests whose trees received full protection from a program such as REDD could, over decades, lose carbon stocks through the ripple effects of bushmeat hunting on species interactions. Actions that shelter trees can also conserve certain smaller organisms, a phenomenon known as the "umbrella species effect." But this trickle-down protection does not extend to large-bodied vertebrates that are directly exploited. Mitigation strategies may help reduce deforestation emissions over the short term, but they must also conserve tropical animals in order to truly keep carbon on the ground into the future.

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Climate Engineering Vulnerable to Disruption

G. C. HEGERL AND S. SOLOMON ("RISKS OF climate engineering," Perspectives, 21 August, p. 955) point out that attempting to engineer climate by decreasing incoming sunlight carries serious risks to the planetary hydrological system. But another set of risks has not been seriously addressed and may be much more consequential. Over the long haul, any kind of climate engineering would have to be sustained, and indeed increased in intensity because of continued rise in greenhouse gas concentrations. Any extended disruption, even for a few years, could lead to catastrophically large and rapid climate variations. Disruptions could arise from any number of societal factors: financial meltdown, political upheaval, environmental disasters, war, or simply loss of interest by the funding public. If climate engineering is seriously considered, it will be necessary to carefully address the likelihood, impacts, and possible responses to such disruptions.

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Conserve Livestock Genetic Resources, Too

M. S. SWAMINATHAN ("GENE BANKS FOR A warming planet," Editorial, 31 July, p. 517) delivered a convincing argument for strengthening and expanding gene banks and other mechanisms for conservation of the biodiversity of agricultural plants, particularly in the context of climate change. This call to action should be echoed for animal genetic resources for food and agriculture. Similar to plants, animal genetic resources are being lost at an alarming rate. The Food and Agriculture Organization of the United Nations (1) has

reported that at least 20% of existing livestock breeds risk extinction, with insufficient data on an additional 30%. Furthermore, largely due to the need for cryoconservation of animal germplasm when stored ex situ and the associated costs and technical expertise required, gene banks for livestock are relatively rare, especially in developing countries.

Although livestock contribute to climate change (2), they will also play an integral role in allowing mankind to withstand its effects. Certain breeds are adapted to marginal areas that are too dry or have other ecological features that render them inhospitable for other forms of agriculture. Other breeds are resistant to endemic diseases that may expand their territory as a result of climate change. The selection of these animals for survival in harsh environments rather than for high productivity has made them more valuable for the future but more vulnerable in the present economic conditions. Without conservation programs, the risk of losing the unique alleles and other genetic variations that underlie this adaptation is great. International collaboration is urgently needed to ensure their conservation.

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Teaching, Not Testing, for Scientific Vision

DAVID LUBINSKI AND CAMILLA BENBOW ARE certainly correct that "Science needs kids with vision" (C. Holden, *News of the Week*, 4 September, p. 1190), but I take issue with their position that we must therefore test visualization as a talent. Our group, too, has demonstrated that use of visualization skills in scientific problem-solving is directly correlated with success as a scientist (1), but we have also demonstrated that crafts and arts skills correlate just as highly (2). Crafts and arts skills also correlate very strongly with visualization ability (1, 3). From these data, we conclude that visualization ability is not an innate talent, but a skill that anyone can learn through the practice of visual arts, crafts, and model building (1, 3). In fact, numerous studies [e.g., (4–7)] show that science

and engineering students who initially test poorly on visualization tests (many of them women) and are subsequently given mechanical or artistic drawing training improve dramatically on retest and perform better overall in their science and engineering coursework. So rather than wasting money using visual tests to search for "Edisons and Fords" overlooked by other standardized tests, I suggest teaching arts and crafts to improve the manipulative and visualization skills of all students (especially women), thereby enlarging the pool of potential innovators.

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CORRECTIONS AND CLARIFICATIONS

News of the Week: "Paper retracted following genome data breach" by C. Holden (18 September, p. 1486). Michael Miller is at the University of Minnesota in Minneapolis and not at the University of California, Santa Barbara.

News Focus: "A cure for euthanasia?" by D. Grimm (18 September, p. 1490). On page 1493, the statement "Briggs says ACC&D's lack of funding slowed U.S. Food and Drug Administration approval" should instead be "ACC&D's Briggs says lack of funding slowed U.S. Food and Drug Administration approval."

Brevia: "30,000-year-old wild flax fibers," by E. Kvavadze et al. (11 September, p. 1359). The fiber length unit of measure was incorrect. The correct sentence is "The complete fibers are long (>200 μm) and composed of segments of smaller lengths."

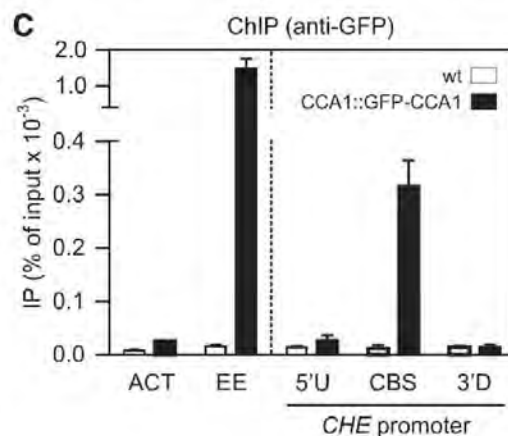
Reports: "Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate" by N.C. Hait et al. (4 September, p. 1254). In Fig. 4A, the second group of columns should be labeled "V5-SphK2," not "H3-K9acH2."

Reports: "Spectroscopic fingerprint of phase-incoherent superconductivity in the underdoped $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_{8+\delta}$ " by J. Lee et al. (28 August, p. 1099). The title should have been "Spectroscopic fingerprint of phase-incoherent superconductivity in the cuprate pseudogap state."

Reports: "Coupling mechanics to charge transport in carbon nanotube mechanical resonators" by B. Lassagne et al. (28 August, p. 1107). The name of the fourth author was incorrect. It should be Daniel García-Sánchez. The name has been corrected in the HTML version online.

Letters: "Creationists made me do it" by P. J. Keeling (21 August, p. 945). The book in the cartoon should have been titled "On the Origin of Species."

Reports: "A functional genomics approach reveals CHE as a component of the *Arabidopsis* circadian clock" by J. L. Pruneda-Paz et al. (13 March, p. 1481). In Fig. 3C, several bars at the right of the figure were missing. The corrected figure is shown here.



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EVOLUTION

A Fresh Theoretical Framework

Jay Odenbaugh

Peter Godfrey-Smith's *Darwinian Populations and Natural Selection* is a dense and deep work on the foundations of evolutionary biology. Evolutionary biologists tell us that evolution by natural selection occurs when a few ingredients are present—specifically, when there is variation with respect to a trait, those variants differ in the numbers of offspring produced, and this variation is heritable to some degree. Unfortunately, as Godfrey-Smith argues, this recipe is far too simple, and even more complicated versions such as the replicator approach offered by Richard Dawkins suffer serious flaws. This “classical recipe,” for example, ignores the fact that for some organisms numbers of offspring don’t necessarily determine reproductive success (“fitness”) whereas rates of population growth, age structure, or variation in expected numbers of offspring do. Likewise, natural selection and patterns of heredity can “cancel” each other out, leaving no evolutionary change. The concept of Dawkins’s replicators—those entities that interact with like entities and of which copies are made—presupposes that there can be no reproduction without replication, which is false when we have continuously varying traits evolving by natural selection. Thus, our standard models for understanding what evolution by natural selection is are just too simple.

This is where Godfrey-Smith’s notion of a Darwinian population comes to the fore. A Darwinian population “is a population—a collection of particular things—that has the capacity to undergo evolution by natural selection. A ‘Darwinian individual’ is any member of such a population.” By itself, this is not terribly helpful, but Godfrey-Smith (a philosopher at Harvard University) provides a spatial framework for characterizing what he terms minimal, paradigm, and marginal Darwinian populations. Although he mentions a variety of parameters, he focuses on three: the reliability of inheritance between parent and offspring (H); continuity in fitness, the degree to which small changes in trait val-

ues are associated with small changes in fitness values (C); and the dependence of reproductive differences on “intrinsic” or non-relational traits (S). We can imagine a space with just those dimensions and then locate different Darwinian populations therein. For example, low values of each variable gives us marginal populations and high values of each gives us paradigm populations. Godfrey-Smith then uses this spatial framework (along with others concerning reproduction) to understand controversies concerning the nature of random genetic drift, levels of selection, major transitions in evolution (such as the appearance of multicellular organisms), and cultural evolution. Let’s consider what light Godfrey-Smith’s framework shines on some of these topics.

Among philosophers and biologists, there has been a debate raging over the nature of random genetic drift. Some argue that drift is a “force” or a causal process like natural selection, mutation, and migration, whereas others understand it as a category representing our demographic ignorance. Godfrey-Smith argues we should reject both options. Consider identical twins standing near one another when one unlucky brother is struck by lightning and subsequently dies. Note that in this instance and in many others, individuals such as this one will have low values of C because large differences in their reproductive output result from very small changes in their characteristics (in this case, location).



Exemplar marginal. Slime molds of the amoeba *Dictyostelium discoideum*.

Likewise, this sort of individual will have low S values because its reproductive output depends on much other than its own characteristics. Thus, drift is not a “force” or a label for we know not what; rather it concerns where one is in the space of Darwinian populations. With regard to the levels of selection controversies, Godfrey-Smith argues that many supposed instances of group selection involve marginal Darwinian populations at best. For example, in cases where selection occurs in neighborhoods, there are no causally cohesive groups for selection to operate on. With regard to evolutionary transitions, he notes that often the formation of new biological individuals involves marginal Darwinian populations moving to paradigmatic ones and the parts of such populations (that is, the lower-level entities) moving from paradigmatic ones to marginal ones—a process he terms “de-Darwinizing.”

Godfrey-Smith then turns to complications regarding the notion of reproduction, which is one component of his Darwinian populations. Here he introduces three different concepts of reproduction: collective, simple, and scaffolded reproducers. A collective reproducer is one “with parts that themselves have the capacity to reproduce.” A simple reproducer reproduces largely with its own “machinery.” And a scaffolded reproducer gets reproduced as part of a larger, reproducing entity. These distinctions are skillfully employed. For example, contrary to Richard Dawkins, many instances of genic selection are instances of scaffolded reproduction of genes by cells, and evolutionary models are ultimately representing selection of organisms via their genetic properties. Often (though not always), when we treat genes as evolutionary units we imbue evolutionary biology with an “agential” framework involving agents, goals, strategies, and purposes that can corrupt the foundations of evolutionary biology.

Darwinian Populations and Natural Selection raises difficult questions as well. Godfrey-Smith and others have argued that there is a role in evolutionary biology for “functional” notions. For example, they hold that it makes sense to claim that the heart in humans has the function of circulating blood. However, given the author’s criticism of the “agential” framework and the teleology behind it, is this new work compatible with the old? In addition, although spatial frameworks or state spaces can be exceedingly useful for understanding evolutionary processes, one can ask if they also conceal much of importance. Their use is critically dependent on which dimensions are included

Darwinian Populations and Natural Selection

by Peter Godfrey-Smith

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(and which omitted) and on whether one can “score” those dimensions in plausible ways. Sometimes one wonders whether too much is being omitted and worries that variables like *S* cannot be scored in any object sense. Nevertheless, Godfrey-Smith’s book fruitfully forces us to think in new ways about evolution and natural selection.

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PUBLIC HEALTH

Toward a Malaria Vaccine

Brian Greenwood

Vaccination is a highly effective public health intervention, especially in communities where urgently accessing treatment is difficult. Therefore, it is not surprising that major efforts are being made to develop vaccines against the “big three” infectious diseases of the developing world: HIV, tuberculosis, and malaria. Creating an effective malaria vaccine has proved to be a major challenge. The story of this as yet unsuccessful quest is the subject of Irwin Sherman’s excellent *The Elusive Malaria Vaccine*.

A lot is known about the early history of malaria research because letters and diaries written by the key protagonists have survived for use by historians. In the era of the ephemeral e-mail, following the sequence of events underlying major scientific discoveries is going to be more difficult. I wonder how many of the scientists currently engaged in malaria vaccine development keep a diary or copies of their correspondence. In the future, establishing the story behind a major scientific breakthrough will often need to come from personal contacts with the main protagonists by an impartial observer. Sherman (an emeritus professor of biology at the University of California, Riverside) is very well qualified to take on this role in relation to the development of malaria vaccines as he has worked on the immunology and pathogenesis of malaria for 50 years. He knows many of the key investigators (whose brief biographies are interspersed throughout the text, helping to break up some fairly detailed tech-

nical sections) but is not tied to any particular vaccine candidate. Sherman has previously edited two volumes on malaria biology (1, 2); here he offers a very balanced account of the malaria vaccine story so far.

Selecting the audience for a book such as this is difficult: Should one write for the general reader, for the reader with a good scientific background but little knowledge of malaria, or for the malaria specialist? Sherman opted for a compromise, and he has largely succeeded, as all these readers will find much of interest in the book. However, there are sections that readers in each group will want to skip. For example, the early chapters describe the histories of malaria and vaccination in general, which have been well covered elsewhere. It is only in the sixth chapter, “Dreams about vaccines,” that the particular story of the efforts to develop a malaria vaccine reaches its stride.

The author covers the immunology underlying vaccine development well. Some of his account is quite technical—for example, the sections on mechanisms of nonspecific immunity and cytokine interaction—and may be too detailed for the general reader. The stron-

gest part of the book describes in considerable detail the twists and turns that have taken place in vaccine development since the first successful attempt at vaccination against malaria (in birds) was undertaken by the brothers Edmond and Etienne Sergent 100 years ago (3). This provides a unique record of a remarkable story by someone who has observed much of it at first hand.

The author views the malaria vaccine story from the perspective of a laboratory scientist based in the United States. Having spent most of my career working on malaria in Africa, I would like to have seen more attention given to the ways in which investments in malaria vaccine development during the past 10 years have supported the establishment and growth of research centers in areas where malaria remains endemic. These centers have made it possible to carry out vaccine trials meeting the highest international standards. Malaria vaccine research has contributed to advances in epidemiology, immunology, and molecular biology in these malaria-endemic areas.



Intended target. Malaria parasites (*Plasmodium*) emerging from a red blood cell (SEM image).

Development of a highly effective malaria vaccine would be a major advance that could lead to a Nobel prize for its discoverer(s). It is therefore not surprising that malaria vaccine research has attracted some strong personalities and even some corrupt ones—exemplified by the notorious malfeasance of James Erickson while serving as chief technical officer for the U.S. Agency for International Development’s Malaria Immunology and Vaccine Research program during the 1980s. Most scientists, myself included, enjoy reading about such scandals. That is something we should probably not be proud of, but they reflect the passions and rivalries that take place within our own, more modest, scientific circles—which, fortunately, do not usually find their way into the public domain. Sherman deals with these unattractive aspects of malaria vaccine development in a forthright but dispassionate way.

Will we ever have a highly effective human vaccine? Readers of *The Elusive Malaria Vaccine* are likely to conclude that before too long we will have one or more partially effective malaria vaccines such as RTS,S, which is currently undergoing a phase III trial. But when a highly effective vaccine will emerge is much more difficult to predict. Sherman’s book is full of quotations by those who should have known better that a highly effective malaria vaccine was just around the corner. This reviewer is not going to add his name to that list.

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The Elusive Malaria Vaccine: Miracle or Mirage?

by Irwin W. Sherman

ASM Press, Washington, DC,
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Balancing Innovation and Access: Patent Challenges Tip the Scales

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Improvements in pharmaceutical research and development (R&D) depend on product innovation. But the number of new compounds approved annually by the U.S. Food and Drug Administration (FDA) has fallen from an average of 35 in 1996–2001 to 20 in 2002–07 (1). This decline stems from several factors (2); however, one particular U.S. regulation—the Paragraph IV patent challenge—is increasingly stifling new drug innovation and deserves our attention.

Paragraph IV Patent Challenges

Although the U.S. patent system has been criticized, particularly when it interferes with access to medications (3), patents are widely used, and there is little debate that they are important for spurring drug innovation (4). In a market system of pharmaceutical innovation, industry revenues support continued R&D, and patents support revenues. The estimated average R&D cost of a new drug brought to market in 2000 exceeded \$800 million (5). Because drug companies are making substantial investments with no certainty about outcomes, they rely on patent-protected revenues to recoup their R&D expenditures (6). Although drugs newly approved by the FDA are awarded 5 years of “data exclusivity” during which generic versions may not be marketed (7), effective patents can offer longer protection by blocking substitute products beyond the 5-year data exclusivity period.

Nevertheless, companies that produce generic drugs can challenge such patents, beginning the process of competing with brand-name drugs after only 4 years (7). To market a generic version, the law requires a company to file an Abbreviated New Drug Application (ANDA) with the FDA that specifies how the generic version relates to the brand-name drug and its patents (7). Paragraph IV permits generic-producing companies to “challenge” each patent associated with the brand-name drug, stating either that

(i) the patent is invalid or (ii) the ANDA does not infringe the patent (7). Although ANDA approvals involving patent challenges generally take from 2 to 3 years (8), the first Paragraph IV applicant is offered 180 days, should their challenge succeed, during which no other generic-producing company may enter the market (7). This incentive to challenge patents allows the successful challenger, without fear of cheaper generics competition, to reap large dividends by pricing just below the brand-name drug (9), earning on average \$60 million per drug (10).

Congress’s Intended Balance

The ANDA and Paragraph IV challenge are elements of the Drug Price Competition and Patent Term Restoration Act of 1984 (Public Law 98-417, also known as the “Hatch-Waxman Act”). Congress originally intended to strike a balance between two conflicting, but related, policy objectives: ensuring timely, affordable access to drugs, by allowing for expedited FDA approval of generic drugs, and encouraging drug innovation, by restoring some years of patent protection that are lost by firms during the average 1 to 2 years spent in the FDA approval process (8, 11). But the balance is tipping away from the incentives needed to support innovation (12, 13). The Congressional Budget Office estimated that increased competition from generic drugs resulted in a 12% loss in revenues on sales of brand-name drugs (14). This loss exceeded companies’ gains from the patent extensions awarded to them (11).

Sapping Innovation Incentives

These challenges diminish industry revenues and profits, contributing to the current crisis in industry R&D pipelines (15). Because the costs of challenging a patent are a relatively small \$5 million (16) compared with the large average potential payoff of \$60 million in the first 180 days alone (10), generic-producing firms have begun to engage in “prospecting” by filing numerous ANDAs with Paragraph IV challenges. Simple arithmetic suggests that generic-manufacturing companies only need to win a fraction of these to make a prospecting strategy successful. And they are successful: The Federal Trade Commission showed that 72% of Paragraph IV challenges

Development of new but costly pharmaceuticals is put at risk by U.S. law that emboldens generics competition.

PARAGRAPH IV CHALLENGES

Year	Suits filed	Branded drugs involved
2001	35	16
2002	33	16
2003	96	38
2004	90	21
2005	81	33
2006	87	32
2007	162	44
2008	165	43

Paragraph IV Challenges. Since 2001, companies have filed 749 lawsuits responding to Paragraph IV challenges on 243 unique brand-name products (20).

filed from 1992 to 2000 resulted in litigation, with the generic drug challenger winning 42% (8). Moreover, shifts in judicial interpretation of patent laws over the last 3 years have made these challenges easier to launch and win (17).

Paragraph IV challenge suits have been increasing and nearly doubled from 2006 to 2007 (see table, above). Although the upward trend can be traced to legal and regulatory changes made during the last decade (18), the spike in 2007 resulted from a combination of more drugs losing data exclusivity (19) and additional generic-producing companies filing challenges (20). Moreover, the drugs being challenged increasingly have revenues below \$100 million, which shows that “blockbusters” are no longer the only target (19).

Both the type and timing of new products offered to the public are affected. Pharmaceutical companies targeted with Paragraph IV challenges are responding by introducing new products to bolster their at-risk revenues, but these “countering” drugs are more than 90% more likely to be a reformulation or a “next-generation” product, at best marginal improvements over present-day pharmaceuticals, as compared with all other product introductions (12). So, although these new products reach the public sooner, they are much less likely to offer improvement over previous products.

Pharmaceutical firms’ revenues are being severely affected by these challenges. For example, after Merck’s osteoporosis drug

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Fosamax lost a Paragraph IV challenge, Teva Pharmaceuticals began selling its generic alendronate in February 2008, ~4 years before the Fosamax patents were due to expire (21). Fosamax sales subsequently plunged from \$3 billion in 2007 to \$1.5 billion in 2008 (22). Industry-wide, Teva's portfolio of 160 pending ANDAs in 2007 included 92 Paragraph IV challenges, putting at risk over \$100 billion in sales (23).

These challenges are occurring earlier in the life-cycle of brand-name drugs (11, 19), especially for "blockbusters" (24, 25), and successful challenges shorten patent life (19). Thus, without policy intervention, the effective life of key patents will continue to decline, which further compresses the pay-back period during which brand-name firms can recoup R&D investments. The Fosamax case illustrates the dilemma: Although a cheaper version was available to consumers sooner, Merck's revenue loss is roughly equivalent to the expense of bringing two new drugs to market, or nearly 10% of all new drugs introduced in the United States in 2008 (5, 26). Society ought to be concerned about less pharmaceutical innovation, because research shows it is positively related to life expectancy (27) and to lower nondrug medical spending of all types (28).

Evaluation and Prescription

Although the Hatch-Waxman Act was enacted 25 years ago with laudable intentions, lawmakers should now consider altering the length of data exclusivity awards, as research shows that the 5-year allowance is generally insufficient to recoup R&D costs (11, 29). The U.S. National Academies recommends at least doubling the duration

of data exclusivity (29), bringing the U.S. closer to allowances awarded in the European Union, Japan, and Canada (see table, below). Critically, Congress is now debating this issue with respect to biologic drugs: A rule allowing 12-year data exclusivity was reported out of committee (ready to be placed on the legislative calendar) in August despite the White House's support for 7 years (30).

Exclusivity could be extended for "first-in-class" and high-risk, high-necessity drugs, such as preventive medicine for Alzheimer's disease or osteoarthritis (31). Preliminary investigations could allow regulators to create test cases with increased incentives for a subset of drugs across therapeutic categories. Policy-makers could use incentives to encourage private investments in research to complement public research (such as at the NIH) or to offer increased exclusivity to curative and preventive, as compared with palliative, drugs. Auctions could allow companies to bid on specific research projects in return for extended data or market exclusivity. But programs' complexity and administrative burdens should not outweigh benefits.

A robust system of market innovation is built on financial incentives (32). Economic research shows that there is a market failure, and it requires innovative solutions. At a minimum, we should all note that Paragraph IV challenges are contributing to this failure when discussing the future of health care and long-term access to new treatments.

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CHANGES IN NATIONAL DATA EXCLUSIVITY PROTECTION

Region	Data exclusivity regime (years)		Total sales (millions of \$)	Total R&D (millions of \$)
	Former	Present		
European Union	6 to 10	10 + 1	43,661	8,170
Canada	5	8 + ½	6,693	612
Japan	6	8 + 2	9,089	954
United States	5	5	185,209	36,608

Changes in national data exclusivity protection, along with corresponding pharmaceutical sales and R&D expenditures (33–36). The 6- to 10-year range was the result of differences between the national regulatory regimes of E.U. members. These differences in national regimes are the motivation behind the E.U. harmonization to the present 10 years. The +1, +½, and +2 are extensions beyond the base periods that are available to applicants. For example, the +1 in the European Union is available to applicants if they pursue another treatment or therapeutic category for an already approved drug. Sales in the European Union, Canada, and Japan are by United States–owned firms and U.S. divisions of foreign-owned firms. U.S. sales include both United States–owned and foreign-owned firms' sales in the United States. These figures demonstrate the relative size of the relevant markets in which U.S. firms compete. R&D figures reflect investments only by major industrial pharmaceutical companies (e.g., not smaller start-up firms or the government).

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NEUROSCIENCE

The Speaking Brain

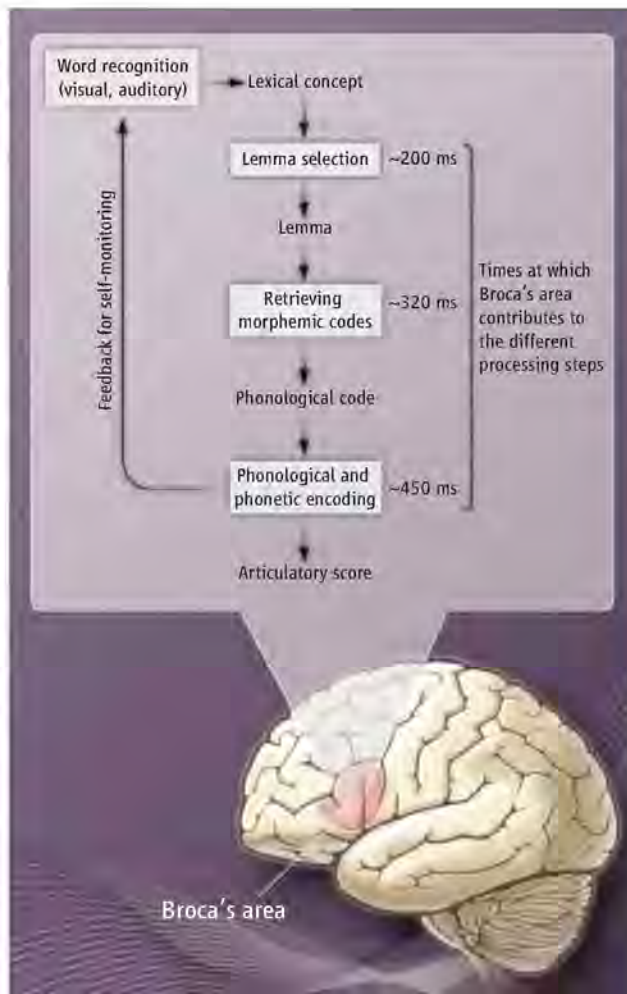
Peter Hagoort^{1,2} and Willem J. M. Levelt¹

How does intention to speak become the action of speaking? It involves the generation of a preverbal message that is tailored to the requirements of a particular language, and through a series of steps, the message is transformed into a linear sequence of speech sounds (1, 2). These steps include retrieving different kinds of information from memory (semantic, syntactic, and phonological), and combining them into larger structures, a process called unification. Despite general agreement about the steps that connect intention to articulation, there is no consensus about their temporal profile or the role of feedback from later steps (3, 4). In addition, since the discovery by the French physician Pierre Paul Broca (in 1865) of the role of the left inferior frontal cortex in speaking, relatively little progress has been made in understanding the neural infrastructure that supports speech production (5). One reason is that the characteristics of natural language are uniquely human, and thus the neurobiology of language lacks an adequate animal model. But on page 445 of this issue, Sahin *et al.* (6) demonstrate, by recording neuronal activity in the human brain, that different kinds of linguistic information are indeed sequentially processed within Broca's area.

Sahin *et al.* had the unique opportunity to record from three patients with epilepsy during presurgical preparation. Depth electrodes were implanted in Broca's area and the anterior temporal cortex, and local field potentials were recorded while the patients were engaged in a language production task. The subjects were asked either to read silently words presented on a screen, or to silently produce the inflected form of the presented

nouns and verbs in accordance with the syntactic requirements imposed by a short sentence fragment (e.g., Yesterday they... walked). This latter process has two components (see the figure). One is to determine the correct tense of the target word and to generate (for regular inflections) or retrieve (for irregular inflections) the correct morphological form. The other is the generation of the concomitant phonological code and preparation for articulation.

Particularly in Broca's area, more specifically Brodmann area 45, a clear triphasic local field potential response was observed. At about 200 ms after presentation of the word, word identification had taken place, with a stronger response for low-frequency words than for high-frequency words. Morphological composition and retrieval for nouns and verbs happened at around 320 ms. Finally, at about 450 ms, phonological encoding had been completed. All these operations



Recordings of electrical activity in the human brain reveal the fine-tuned, stepwise neuronal processing of language and speech.

From intention to articulation. Shown is an adapted version of the lexical encoding model for speech production (2), specifying steps in the paradigm used by Sahin *et al.* Based on the visual input, a lemma is selected that specifies the syntactic features of a lexical concept. For instance, for the lemma *horse*, it specifies that it is a count noun. In addition, the morphemic codes are retrieved. For instance, when the speaker wants to produce the plural form of *horse*, the codes for both the stem and the plural suffix are retrieved. Next, the phonological codes for each morpheme are retrieved, combined, and transformed into a motor command to the articulatory system. The approximate times (in milliseconds) at which Broca's area contributes to the different processing steps are shown. The late (i.e., at 500 to 600 ms) monophasic component observed in the temporal lobe (6) might reflect self-monitoring of the speech output.

were not only temporally separated, but also spatially segregated at a scale of only a few millimeters, which is below the effective spatial resolution of standard functional magnetic resonance imaging of brain activity.

These data are relevant for both cognitive models of speech production and for accounts on the role of Broca's area. The time course is clear evidence for the sequentiality of different access and unification operations in speaking, and is consistent with the few estimates in the literature (7, 8). Moreover, both the anatomical and the temporal segregation of word-encoding operations in Broca's area are in line with the view that this region is involved with each of these encoding operations and their unification over time. Feedback operations among these processes cannot be excluded. However, the fine-grained temporal and spatial separation of these steps suggests that we are witnessing the "first go" process at work here.

Both functional magnetic resonance imaging and lesion studies have shown that Broca's area is also involved in processing inflectional morphology during comprehension (9). In combination with the findings of Sahin *et al.*, this suggests that Broca's area is recruited during both language production and comprehension. Whether these recruitments can be separated at the scale of the microcircuitry within Broca's area remains to be seen.

Broca's area has been proposed to have a more specialized role in language processing—facilitating linguistically motivated operations of syntactic movement (10) and processing hierarchical structures (11). The

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results of Sahin *et al.* indicate that the role of Broca's area is not so limited, but should be characterized in more general terms. It is likely involved in unification operations at the word and sentence level, in connection with temporal regions that are crucial for memory retrieval (12).

As is known for neurons in the visual cortex (13), the specific contribution of Broca's area may well vary with time, as a consequence of the different dynamic cortical networks in which it is embedded at different time slices. This fits well with the finding that Broca's area is not language specific, but is

also recruited in the service of other cognitive domains, such as music (14) and action (15), and with the finding that its contribution to language processing crosses the boundaries of semantics, syntax, and phonology.

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PALEONTOLOGY

Becoming *T. rex*

James Clark

Gigantic, ferocious, long-dead animals like *Tyrannosaurus rex* never fail to capture people's attention, and the discovery of a new tyrannosaur—giant or otherwise—is always big news. On page 418 of this issue, Sereno *et al.* (1) report on a spectacular skeleton of a new genus and species near the ancestry of the group including *T. rex* and its closest relatives, the Tyrannosauridae. At an estimated 3 m total length, *Raptorex kriegsteini* is much smaller than the largest *T. rex* [12.8 m long (2)] and other tyrannosaurids, but has several key features previously known only in this family. *Raptorex* thus provides a glimpse at how tyrannosaurids evolved.

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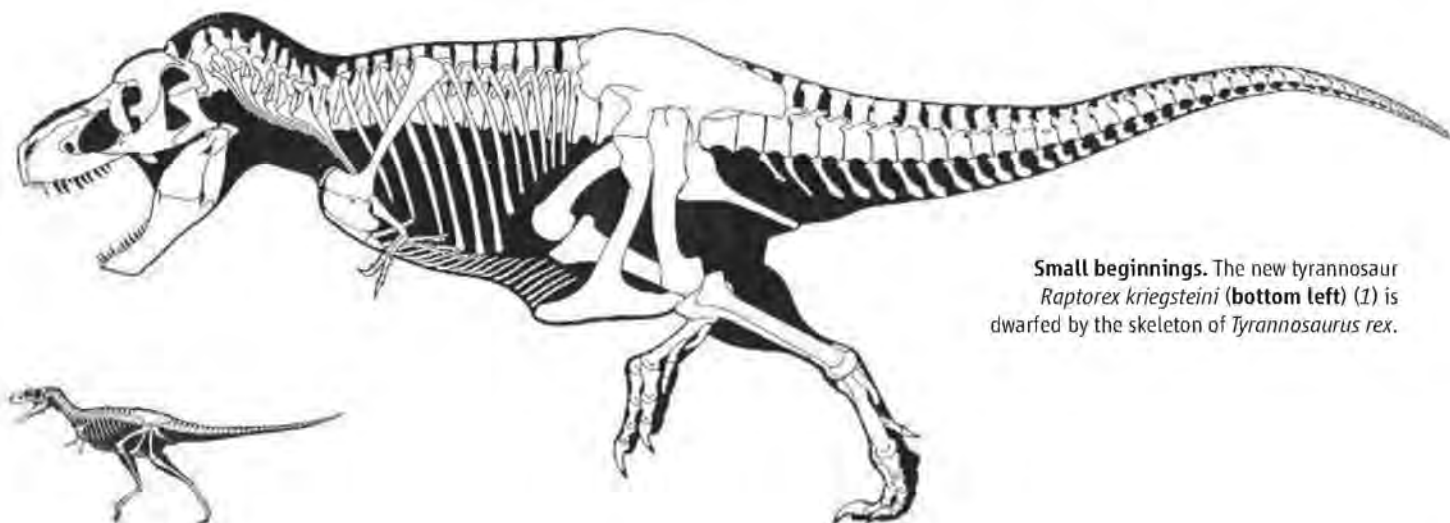
Fossils preserved in the rock with *Raptorex* point strongly to its origin from the beds at the bottom of the Jehol Group in northeastern China, although the locality remains unknown. The Jehol Group fossil beds (3) are famous for preserving dinosaurs with feathers in their thin-bedded shales, including the basal tyrannosaur *Dilong* (4), but the skeletons are usually crushed into two dimensions, and structures such as the skull are difficult to study. Fortunately, a series of beds in the lowest part of the Jehol Group yields exquisitely preserved, uncrushed skeletons, albeit without any soft tissues.

The *Raptorex* specimen was purchased a few years ago by Henry J. Kriegstein at the Tucson Gem and Mineral Show (5), a venue notorious for the sale of illegally collected fossils, such as the famous *Archaeoraptor* chimera from the Jehol Group (6). Kriegstein approached Sereno

A small tyrannosaur from the Early Cretaceous sheds light on the origin of predatory features of *Tyrannosaurus rex*.

with the fossil, and Sereno agreed to describe it on the condition that it would be deposited in a collection in China (5). Although the fossil is currently with Sereno in Chicago, the specimen will be deposited in the Long Hao Institute of Geology and Paleontology in Hohhot, Inner Mongolia. Lin Tan of that institute is a coauthor of the paper, along with Kriegstein.

What to do with “hot” specimens is a conundrum for scientists. Such specimens almost always lack reliable locality data and therefore information about the sediments in which they were preserved. Stolen fossils can preserve data about the anatomy of a new or poorly known species, but enriching thieves or their fences is no more ethical for a fossil than for a car or a Grecian statue. The naming of a new ankylosaur, *Minotaurasaurus ramachandrani* (7), was strongly criticized (8), because the fossil was almost certainly



Small beginnings. The new tyrannosaur *Raptorex kriegsteini* (bottom left) (1) is dwarfed by the skeleton of *Tyrannosaurus rex*.

exported illegally from Mongolia or China. An associate of the authors purchased the specimen after they identified it, and *Mino-taurasaurus* remains in the United States.

In the case of *Raptorex*, the fossil had already been purchased and mistakenly identified as a baby *Tarbosaurus* (a close relative of *Tyrannosaurus* from Asia that would also require permits) before Kriegstein brought it to Sereno. The agreement to return the fossil to China should be applauded.

The fossil record of dinosaurs is strongly biased toward those living near the end of the Mesozoic Era (9); we thus know much more about dinosaurs such as *T. rex* and *Tarbosaurus* from the Late Cretaceous (99.6 to 65.95 million years ago) than we do about their relatives from the Early Cretaceous (145.5 to 99.6 million years ago) and Jurassic (201.6 to 145.5 million years ago).

Twenty years ago, tyrannosaurs were thought to be related to other large theropod dinosaurs, such as *Allosaurus* of the Late Jurassic, and almost nothing was known of early, basal tyrannosaurs—that is, those outside the Tyrannosauridae but closer to them than to other groups of theropods. Since then, it has become clear that tyrannosaurs are instead members of a group of

theropod dinosaurs called coelurosaur (10), a group from which birds also evolved. The publication of *Raptorex* brings to five the number of basal species of tyrannosaurs from the Jurassic and Early Cretaceous. Their relatively small size confirms that tyrannosaurs evolved from small-bodied species (11), as did other coelurosaurians, and then became larger in the Late Cretaceous, independent of more basal theropods like allosaurids.

The importance of *Raptorex* is that it shares with tyrannosaurids several signature features previously unknown in basal tyrannosaurs, such as a large head in proportion to its body and small arms. These features thus evolved before tyrannosaurs became large. They therefore represent discrete evolutionary novelties that appeared somewhere between basal tyrannosaurs on the one hand and *Raptorex* and tyrannosaurids on the other, rather than being incipiently developed but unexpressed until tyrannosaurs reached larger body size.

One of the frustrations of paleontology is that a discovery representing a major leap in understanding a group must be understood in the context of the rest of the group, much of which may be poorly known. With *Rap-*

torex, the problem is that most basal tyrannosaur fossils are fragmentary. For example, the recently discovered *Xiongguanlong* from Early Cretaceous beds in western China (12) might also have a relatively large head and short arms, but the skeleton is too poorly known to be sure. The next goal in tyrannosaur phylogeny must be to find more complete material of other basal tyrannosaurs.

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MATERIALS SCIENCE

A Ball-and-Chain Polymer Model

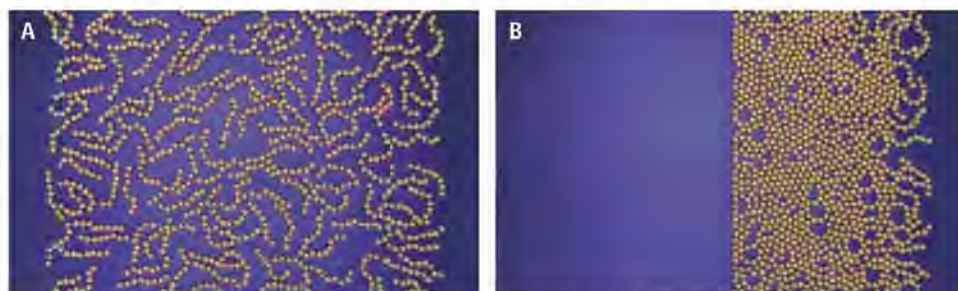
C. J. Olson Reichhardt¹ and L. M. Lopatina^{1,2}

Polymers are long, flexible molecular chains that are ubiquitous in nature and can be found in plastics, rubber, complex fluids, and biological matter (1, 2). A wide diversity of materials owe their properties to the variety of configurations and phases that polymers can adopt, but due to the molecular size scale of the polymers it is difficult to probe these configurations directly. On page 408 of this issue, Zou *et al.* (3) describe a macroscopic, millimeter-scale version of a polymer system that not only captures some of the essential aspects of real polymers but also allows the configurations of the system to be observed directly on the individual polymer level, something that cannot be done for real polymers. The ability to capture the behavior of real polymers with a tunable and tractable model system opens an entirely new field of research for polymer science as well as for granular media.

Zou *et al.* consider a granular chain, a system comprising ball chains of the type used as pullcords for a light, cut so that there are M balls per chain (M ranges from 1 to 4096). The chains are placed in a three-dimensional container, and this container is then tapped for a certain number of cycles before the density of the system is measured. The final packing density initially decreases with increasing chain length and then saturates for long chains. To image what is occurring in the interior of the three-dimensional chain

A macroscopic toy model provides a method to visualize directly the complex behavior of real polymers.

assembly, Zou *et al.* use x-ray tomography to provide high-resolution structural information on a small volume of chains. These direct observations show that the final density of the chain packing is affected by the formation of “semiloops” or “arcs” in the chains. Because the angle between two neighbors of a single ball on the chain is limited by the physical construction of the chain, the radius of curvature of the semiloops is limited. As the semiloop approaches the minimum size, it becomes rigid. The semiloops that form



Stuck in a jam. Image from a two-dimensional simulation of granular chains that are each eight monomers long. (A) Initial low-density configuration. (B) Configuration after the packing has been compressed with a wall to the jamming density. Semiloops of the granular chains appear as holes in the packing as described by Zou *et al.*

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during chain packing act as if they are large hollow monomers that can pack together to form a jammed (shear-resisting) configuration. A two-dimensional simulation of granular chains at low chain density, where the system is unjammed (has no resistance to shear) (see the figure, panel A), contains randomly configured chains. At high chain density when the system is jammed (see the figure, panel B), semiloop structures appear. Zou *et al.* revealed that semiloop formation is crucial to the behavior of the granular chain system. The presence of semiloops lowers the jamming density of the system, and this effect is more pronounced in longer chains where there are fewer chains per unit volume.

Real polymer systems undergo a transition to a glassy state below some temperature. It is known that this glass transition temperature increases with polymer length and saturates for long polymers (4). In the granular chain polymer model system, there is no temperature; instead, the inverse density serves as a quantity analogous to temperature. When Zou *et al.* compare the behavior of the inverse density of the granular chains as a function of chain length to the glass transition temperature of a real polymer as a function of polymer length, they find that the behaviors are nearly identical. This remarkable result could imply that the glass transition in real polymers might be a jamming transition governed by the formation of rigid polymer semiloops. It would be extremely interesting to determine whether this type of semiloop actually occurs in real polymer systems. Semiloops should form much more easily than complete loops because the two ends of the polymer do not need to be near each other. If semiloops play a role in real polymer systems, this would be one of the most beautiful examples of where a macroscopic toy system explains the physics on a molecular scale. Such a result would also confirm that the ideas of jamming originally proposed for granular assemblies (5–7) are applicable to a wide variety of molecular systems.

Granular polymers also represent a new form of granular matter and most likely will exhibit new kinds of phenomena that do not occur in real polymers or in granular systems composed of individual grains. As the jamming density of the granular polymer system can be controlled readily by adjusting the length of the granular chains, introducing chains into other systems that exhibit jamming may allow for a much more precise control of the jamming of these materials.

The experimental demonstration by Zou *et al.* that granular polymers can give insight into molecular polymer systems potentially opens

a new avenue of research for polymer science. For example, polymer systems under shear often exhibit very complex dynamics. Perhaps by shearing granular polymers, the microscopic motions of the shearing process can be studied directly, along with other analogous phenomena such as entanglement and polymer mechanical properties (1, 2). One can imagine going even further and creating increasingly intricate granular polymers to model biological polymers, such as proteins or DNA.

PHYSICS

Observing Monopoles in a Magnetic Analog of Ice

Michel J. P. Gingras

Experimental evidence has been found that magnetic poles within metal oxide magnets can be separated.

A bar magnet has a north and south pole, and cutting it in half just creates two new poles, not two separated monopoles. However, a recent theoretical proposal suggested that defects in the spin alignment of certain oxide magnets can create separated effective magnetic monopoles (1). These materials are called spin ices because the lowest-energy orientation of the magnetic spins closely mimics the most stable arrangement of protons in water ice (2). On pages 415 and 411 of this issue, Fennell *et al.* (3) and Morris *et al.* (4) report measurements from neutron-scattering experiments showing that the low-energy excitations in spin ices are reminiscent of Dirac's elementary magnetic monopoles (5) that have so far eluded the searches of high-energy physicists. These dissociated north and south poles diffuse away from each other (6) in these oxides and leave behind a "Dirac string" of reversed spins that can be seen as patterns in the intensity of scattered neutrons.

The materials studied, holmium titanate ($\text{Ho}_2\text{Ti}_2\text{O}_7$) (3) and dysprosium titanate ($\text{Dy}_2\text{Ti}_2\text{O}_7$) (4), are geometrically frustrated ferromagnets (7, 8); they are unusual paramagnets with strong spin-spin correlations (8) that become magnetized in a magnetic

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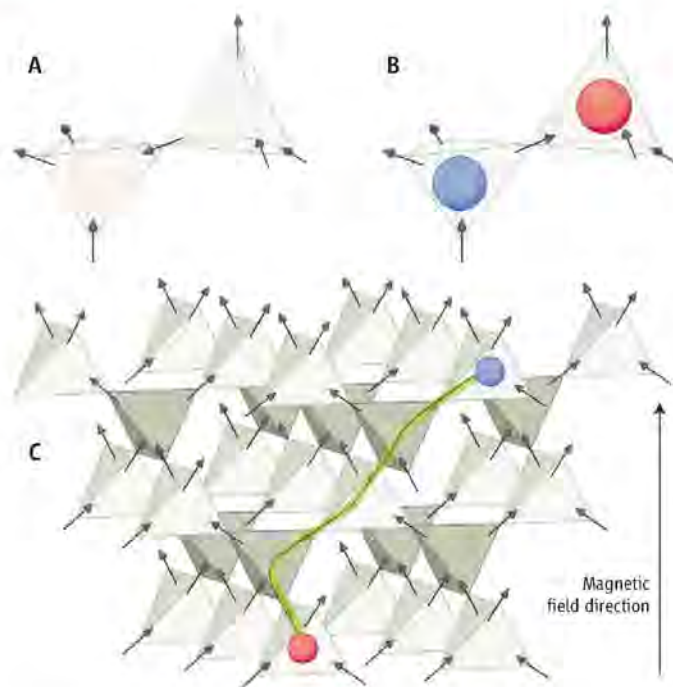
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field. These oxides have a pyrochlore structure; the Ho^{3+} and Dy^{3+} ions within these crystals form a lattice of corner-sharing tetrahedra (see the figure, panel A). The magnetic moments of Ho^{3+} and Dy^{3+} act like two states, or Ising model spins, and are constrained by anisotropic forces to point "in" or "out" of the tetrahedra. The minimum energy condition, or rule, is that there must be two spins pointing "in" and two spins pointing "out" on each tetrahedron (7, 8).

The "two-in, two-out" rule is analogous to the Bernal-Fowler ice rule, which states that for energetic reasons, two protons must be "close" and two protons must be "far" from any given oxygen in common water ice (2). As explained by Pauling (2), the very large number of two-in, two-out configurations in a macroscopic ice sample leads to a measurable residual entropy in ice at low temperatures (9, 10). Correspondingly, $\text{Ho}_2\text{Ti}_2\text{O}_7$ and $\text{Dy}_2\text{Ti}_2\text{O}_7$ also exhibit a residual low-temperature "Pauling entropy" (11).

A tetrahedron fulfilling the two-in, two-out rule amounts to an effective "magnetic charge neutrality" where four (two positive, two negative) magnetic charges cancel out at the center of the tetrahedron (1). A spin flipped by thermal fluctuations creates two defective adjoining tetrahedra—a monopole-antimonopole pair (see the figure, panel B). Once formed, these particles can diffuse away from each other by reversing spins along the path they trace as they separate, which recon-

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Spin excitations creating magnetic monopoles. (A) Spins on two adjacent tetrahedra for magnetic ions in the pyrochlore lattice satisfy a rule that requires two spins pointing in and two spins pointing out, similar to the arrangement of protons in water ice. (B) The reversal of a spin connecting two tetrahedra amounts to the creation of a “monopole” and an “antimonopole” that differ in magnetic “charge.” At finite temperature, monopole-antimonopole pairs are created by thermal fluctuations. The monopoles can separate, leaving behind a “Dirac string” of reversed spins. Their signature was deduced in spin-polarized neutron scattering by Fennell *et al.* in zero applied magnetic field, where the Dirac strings have no preferred orientation. (C) For a magnetic field applied along the crystallographic [100] direction shown, the ground state has the magnetic moments on each tetrahedron pointing with the field, which still maintains the two-in, two-out rule. Two separated tetrahedra, each with a flipped spin (three in, red; three out, blue) leads to a pair of monopoles connected by a string (green) of spins reversed against the field. Morris *et al.* observed a signature for this string in their neutron-scattering data by carefully tuning the applied magnetic field.

stitutes tetrahedra that obey the ice rule along the path (6). In his effort to alter the standard theory of electromagnetism as little as possible (12), Dirac (5) postulated that a monopole-antimonopole pair is connected by an unobservable “string” or tightly wound magnetic solenoids. In spin ices, the “Dirac strings” of reversed spins have direct consequences (3) and are observable (4). Because all tetrahedra, except the two adjoining the north and south poles, fulfill the ice rule, the string is without tension and the energy to separate the poles by an infinite distance is finite (1)—the defects are dissociated, or deconfined.

The monopoles in spin ice act like magnetic charges: They obey analogous electric field laws and exhibit an effective Coulomb’s law for their interaction strength. At zero temperature, the spin-ice state can be viewed as a “vacuum” free of monopoles and is referred to as a “magnetic Coulomb phase” (3). This analogy affords a mathematical framework for calculating the underlying spin correlations of the rare-earth magnetic moments. Thermal fluctuations that create dissociated monopoles are sources of the analogous electric field that modifies the spin correlations (3, 4) and the dissociated monopoles can be used to describe the low-temperature thermodynamic properties of the material (1, 4).

Neutron scattering can probe the “Coulomb phase” nature of the spin-ice state by measuring the spin-spin correlation function, $C(r)$, where r is the distance between spins. In the absence of thermally induced monopoles, $C(r)$ does not decay exponentially with r , as would be the case for a conventional thermally disordered paramagnet. Rather, $C(r)$ is theo-

retically expected to display the same spatial anisotropy and r^{-3} decay as a dipolar interaction. These correlations are manifest in the neutron scattering as “bow-tie” pinch-point singularities at particular neutron-scattering directions of wave vectors $\mathbf{Q}$, which correspond to a “reciprocal space” of the real-space lattice in the crystals. The theoretical argument for the magnetic Coulomb phase (1) is highly compelling, but all previous neutron-scattering experiments, such as those on $\text{Ho}_2\text{Ti}_2\text{O}_7$ (13) and $\text{Dy}_2\text{Ti}_2\text{O}_7$ (14), failed to find an unmistakable signature of the pinch points. Unlike prior studies, Fennell *et al.* performed a polarized neutron-scattering experiment where the scattering signal is separated in two components. The pinch points are clearly revealed in the component where the neutron spin is flipped, confirming the theoretical prediction. The pinch points are obscured in the more intense “non-spin flip” signal, which helps to explain why previous studies were inconclusive.

In their analysis, Fennell *et al.* introduced a parameter that cuts off the pinch-point singularities in reciprocal space, which they associate with the typical length of the Dirac strings. Whereas their experiment was performed in zero magnetic field and did not directly probe those strings, Morris *et al.* applied a magnetic field $\mathbf{B}$ along the [100] crystal direction to induce a magnetically polarized state where the ice rule and the minimum magnetic field energy, or Zeeman energy, are satisfied simultaneously (see the figure, panel C). The magnetic field strength can be tuned near a transition where thermally excited monopole-antimonopole pairs start to proliferate. The resulting flipped spins of the Dirac string are then oriented against the magnetic field direction, with the strings causing cone-like features observable in the scattering inten-

sity pattern. The conic features transform in inclined sheets of scattering when the field direction is tilted away from the [100] direction, in close concordance with the calculations of Morris *et al.* for this state. The specific heat in zero magnetic field can also be described well in terms of a dilute gas of thermally excited monopoles (4).

The demonstration that dissociated monopole-like excitations in spin ices can be observed and manipulated may help guide future studies of similar topological excitations in other exotic condensed matter systems. Of particular interest is the exploration of geometrically frustrated magnetic systems with large quantum mechanical zero-point fluctuations of the magnetic moments away from the classical Ising spin directions (15, 16) enforced in the $\text{Ho}_2\text{Ti}_2\text{O}_7$ and $\text{Dy}_2\text{Ti}_2\text{O}_7$ spin ices considered in (3, 4). Such quantum magnets could provide condensed matter physicists with systems that mimic the physics of quantum electrodynamics.

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MICROBIOLOGY

Fantastic Fixers

Robinson W. Fulweiler

Current global ocean nitrogen budgets do not balance, which suggests that existing models miss or underestimate some contributions to oceanic nitrogen fixation (the conversion of N_2 gas to a biologically usable form of nitrogen) (1, 2). However, recent studies have found higher rates of nitrogen fixation in coastal sediments (3–5) and more abundant nitrogen-fixing organisms in the open ocean (6) than previously observed. This exciting trend continues with the report on page 422 of this issue by Dekas *et al.* (7), who describe a community of archaea and bacteria in deep-sea “cold seep” sediments that can fix nitrogen. The study reveals direct evidence for a previously unknown environment for nitrogen fixation that can deliver biologically usable nitrogen to deep-sea sediments, and provides a link between the carbon, nitrogen, and sulfur cycles.

Cold seeps, like the more familiar hydrothermal vents, are deep-sea ecosystems that depend on chemosynthesis rather than photosynthesis for energy and food production. They fix carbon (typically carbon dioxide or methane) and nutrients into organic matter by oxidizing inorganic molecules. Discovered 25 years ago in the Gulf of Mexico, these systems are sustained by cold water enriched in sulfides and methane (8). Cold seeps are found throughout the oceans, including off the coast of California, where Dekas *et al.* collected their sediment samples from an active cold methane seep (see the first figure).

The authors examined the partnership between anaerobic methane-oxidizing archaea (ANME-2) and anaerobic sulfate-reducing bacteria (*Desulfosarcina/Desulfococcus* or DSS) (9), which together mitigate the release of most of the methane produced in marine sediments. The exact mechanism coupling these two organisms has remained elusive. Furthermore, recent research, based on genetic and preliminary isotope data, suggested that these deep-sea consortia could fix nitrogen (10). However, the presence of genes required for nitrogen fixation does not necessarily mean that nitrogen fixation is an active metabolic process in these organisms. For example, *nif* sequences, which code for the nitrogenase enzyme needed for nitrogen fixation, are ubiquitous in marine sediment and water column bacteria, despite typi-



Sampling the deep. Cold seep ecosystems in the deep ocean are sustained by sulfide and methane emissions from the sea floor. Dekas *et al.* used the Alvin submarine to sample sediments from a seep off the coast of California.

cally low rates of observed nitrogen fixation (11, 12). Thus, Dekas *et al.* sought direct evidence for nitrogen fixation in these consortia.

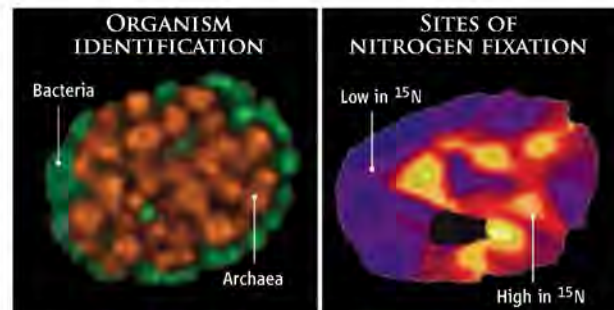
The authors used a powerful tool called multi-isotope imaging mass spectrometry (MIMS) or nanometer secondary ion mass spectrometry (nanoSIMS), which allows the metabolic activity of individual cells to be visualized. For example, Lechene *et al.* recently used this technique to quantify nitrogen fixation by individual bacteria in the gills of a shipworm, *Lyrodus pedicellatus* (13).

Unlike cell cultures, sediment samples are heterogenic environments in which a label can easily go unaccounted. Typically, scientists amend sediment samples with a label and then make bulk measurements of the resulting products, but interpretation of the results relies on a series of assumptions. For exam-

ple, researchers may assume that the label was rapidly and evenly distributed, without really knowing the fate of the label or which organisms were responsible for the detected biogeochemical flux. NanoSIMS enables visualization of the transfer and/or assimilation of a label into a cell, or—as in the case of Dekas *et al.*—into cell aggregates.

Taking the nanoSIMS technique a step further, Dekas *et al.* coupled it to fluorescence in situ hybridization (FISH) to observe assimilation of isotopically labeled ^{15}N from dinitrogen ($^{15}N_2$) gas at the cellular level, thus providing direct evidence of nitrogen fixation in these archaea/bacteria consortia. The authors observed ^{15}N enrichment in the archaea-dominated center of the aggregate and a lower concentration in the bacteria-dominated shell, showing exactly where nitrogen is fixed (see the second figure). Nitrogen fixation ceased when either methane oxidation or sulfate reduction was inhibited, suggesting that both processes are required for successful nitrogen fixation. This observation thus links the cycles of carbon, sulfur, and nitrogen in these sediments.

Nitrogen fixation is energy-intensive, consuming two adenosine triphosphate (ATP) molecules for every electron transferred or ~16 mol of ATP



Working together. As Dekas *et al.* show, consortia of archaea and bacteria in cold seeps can fix nitrogen. In the consortia, archaea incorporate more ^{15}N than do bacteria, indicating that nitrogen fixation takes place in the archaea.

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per mol of N_2 fixed (14). Organisms relying on N_2 tend to have lower growth rates than those that use ammonium. Using ^{15}N assimilation as a proxy for growth, Dekas *et al.* also found that archaea/bacteria consortia grew more slowly while fixing nitrogen than did consortia that were replete with ammonium. However, their respiration rates were similar under either condition; thus, the consortia balance the extra energy required for nitrogen fixation by reducing their growth rate.

The work of Dekas *et al.* is a major breakthrough in understanding nitrogen fixation in deep-sea sediments in general and in cold seeps in particular. Together, the presence of *nif* genes in the archaea and the ability of the archaea/bacteria consortia to fix nitrogen in

lab studies are highly suggestive that they do so in the natural environment. Combining these data on individual cells with larger-scale rate measurements of biogeochemical processes will yield a clearer picture of microbial form and function in the natural environment. Such information may eventually lead to a balanced global nitrogen budget. In the meantime, it will continue to illuminate the tight coupling of microorganisms and elemental nutrient cycles.

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PHYSIOLOGY

Feeding the Clock

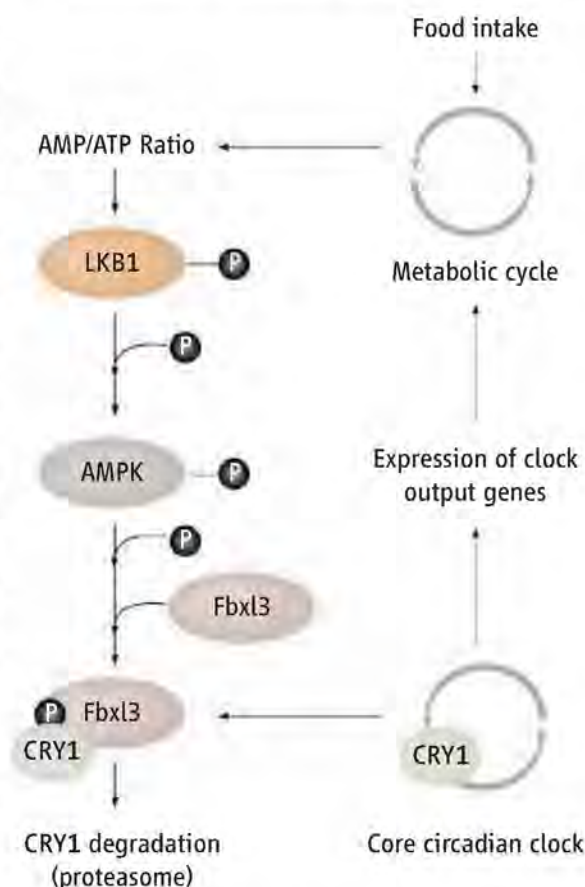
David M. Suter and Ueli Schibler

In mammals, sleeping, feeding, and most other physiological processes are influenced by a circadian system and therefore display daily oscillations. These rhythms are generated by self-sustained and cell-autonomous molecular clocks that exist in virtually all cell types. A “master clock” located in the brain’s suprachiasmatic nucleus (SCN) can synchronize these peripheral clocks, but for many organs, feeding-fasting rhythms are the dominant zeitgebers (timing cues) (1, 2). On page 437 of this issue, Lamia *et al.* propose a molecular mechanism through which metabolic cycles may interact with the circadian clockwork circuitry. They show that an enzyme that responds to nutrient availability—adenosine monophosphate-activated protein kinase (AMPK)—directly phosphorylates the core clock protein cryptochrome 1 (CRY1), thereby marking it for degradation (3).

The core of the mammalian circadian clock is thought to constitute a network of interconnected transcriptional and translational feedback loops that control the oscillatory expression of genes encoding clock components and specific output genes. CRY proteins had previously been described as targets of Fbxl3 (4, 5), a ubiquitin ligase complex that marks proteins for degradation in the protea-

some. However, regulation of CRY-Fbxl3 interactions has remained unknown. Lamia *et al.* identified highly conserved amino acid sequences in CRY1 as potential phosphoryla-

A nutrient-responsive enzyme regulates the stability of a circadian clock component.



tion sites of AMPK. AMPK is activated upon its phosphorylation by protein kinases such as liver kinase B1 (LKB1) or calcium-calmodulin-dependent protein kinase kinase β , which sense the AMP/ATP ratio, a direct readout of the cell’s metabolic state. By using a site-directed mutation analysis of CRY1, serines at position 71 and 280 were identified as critical AMPK phosphorylation sites. When these serines were phosphorylated, the affinity between CRY1 and Fbxl3 increased. In vitro phosphorylation assays and in vivo loss-of-function experiments in cultured mouse embryonic fibroblasts revealed that AMPK is both necessary and sufficient to induce the phosphorylation-dependent degradation of CRY1.

To scrutinize the putative role of AMPK as a food sensor, the authors monitored the expression of circadian clock output genes in mouse embryonic fibroblasts that expressed or lacked AMPK and

Coupled cycles. The AMP/ATP ratio transmits information about the metabolic state of the cell to LKB1, which triggers a cascade of phosphorylation (P) events that eventually targets the clock protein CRY1 for degradation.

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were cultured in different glucose concentrations. The amplitude of circadian oscillations in gene expression correlated to glucose concentrations only in wild-type cells, but not in the absence of AMPK. In mouse liver, the accumulation and nuclear localization of AMPK, as well as the phosphorylation of known AMPK target proteins, oscillated in a circadian manner. Thus, perturbation of nutrient availability—and consequently, of AMPK activity—alters output of the circadian clock.

Although AMPK is an attractive candidate for coupling metabolic and circadian cycles, additional regulators are likely involved. Thus, the ratio of oxidized nicotinamide adenine dinucleotide phosphate (NAD⁺) to its reduced form (NADPH)—which, like the AMP/ATP ratio, constitutes a diagnostic signature of a cell's metabolic state—has been proposed to affect circadian gene expression through diverse mechanisms. At least in vitro, the binding of the heterodimeric core clock transcription factors CLOCK-BMAL1 and NPAS2-BMAL1 to their cognate DNA sequences (so-called E-boxes) is enhanced by NADPH and impaired by NADP⁺ (6). The transcriptional regulatory protein peroxisome proliferator-activated receptor γ (PPAR γ) coactivator 1 α (PGC-1 α), a well-known mediator of glucose and lipid metabolism,

has been proposed to be another important player in connecting metabolism to circadian gene expression. This transcriptional coactivator associates with nuclear receptors of the ROR family and thereby modulates the transcription of the clock genes *Bmal1* and *Rev-erb α* . Finally, the NAD⁺-dependent protein deacetylase sirtuin 1 influences the stability and activity of the core clock components PER2 and BMAL1, respectively (7, 8).

Why are metabolic processes under tight circadian control? A simple explanation arises from the necessity to separate incompatible enzymatic processes within the same cell. Because complete spatial separation of anabolic and catabolic processes is frequently impossible, these have to be gated to different time windows. This necessity is well illustrated by the temporal sequestration of oxidative and reductive phases in yeast by an ultradian respiratory clock. For example, DNA is replicated exclusively in the reductive phase, when the concentration of genotoxic reactive oxygen species generated by mitochondrial respiration is minimal (9). In a yeast mutant in which the reductive phase is too short to allow for the completion of DNA synthesis, the mutation rate increases dramatically (10). In mammals, the master pacemaker in the SCN is phase-entrained primarily by light-

dark cycles and thus cannot readily adapt to altered feeding rhythms. Hence, when food availability changes, nutrient-dependent synchronization cues must dominate the more direct signals from the SCN to maintain proper homeostasis of metabolism in peripheral tissues (1). This could explain the multitude of metabolic phase entrainment cues that synchronize the circadian core clock machinery in metabolically active peripheral organs. A major challenge will be to understand how the multiple nutrient-dependent inputs are integrated so as to maintain coherence between the metabolic state of the organism and the circadian system.

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10.1126/science.1181278

NEUROSCIENCE

How Good Are Neuron Models?

Wulfram Gerstner and Richard Naud

Opinions strongly diverge on what constitutes a good model of a neuron (1–3). Two lines of thought on this have coexisted for a long time: detailed biophysical models (of the style proposed in 1952 by the physiologists Alan Hodgkin and Andrew Huxley) that describe ion channels on the tree-like spatial structure of the neuronal cell (4), and simple “integrate-and-fire” models based on the much older insight that pulsatile electrical activity (known as an action potential or spike) is a threshold process. Electrophysiologists generally prefer the biophysical models, familiar with the notion of ion channels that open and close (and hence, alter neuronal activity) depending on environmental conditions. Theoreticians,

by contrast, typically prefer simple neuron models with few parameters that are amenable to mathematical analysis. Earlier this year, following previous attempts at model comparison on a smaller scale (5), the International Neuroinformatics Coordinating Facility (INCF) launched an international competition (6) that allowed a quantitative comparison of neuron models.

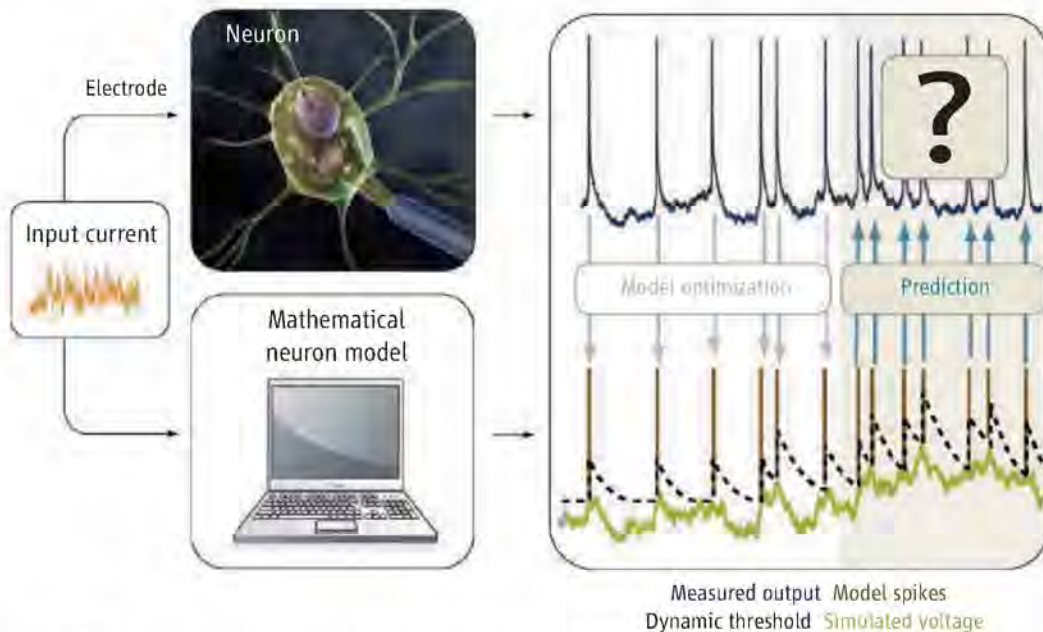
The idea behind the INCF competition is that a good model can predict neuronal activity based on data that were not used for parameter tuning (see the figure). The competition included three in vitro and one in vivo data set. The in vitro data sets were assembled from classical electrophysiological experiments in which random electrical current was injected through an electrode into a pyramidal cell and an interneuron. The task was to predict for 13 (or 9, respectively) repetitions of the same injected current waveform, the exact timing of spikes

A recent competition encouraged modelers to predict neuronal activity. Which neuron model performed the best?

in neuronal electrical activity evoked during a 22-s time span, based on the activity observed during the first 38 s of data collection. The winning submission correctly predicted 59.6% (or 81.6%, respectively) of the spike times of the two neurons, using a simple integrate-and-fire model with a moving threshold (7).

Most threshold models are point neuron models—they neglect dendritic morphology and reduce the neuron to an extensionless mathematical construct. However, the INCF competition included as a third challenge a double-electrode experiment, in which current injection into the neuronal cell body (soma) was combined with current injection into the apical dendrite located about 600 to 700 μ m from the soma, enabling an intricate interplay between somatic and dendritic spike activity (8). Surprisingly, the best performance was achieved by a variant of a threshold model, enriched with two equations for the dendrite.

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Predictions. The same fluctuating input current is injected into a live neuron and a model neuron. The first few seconds of voltage time course (dark blue) are used to optimize the parameters of the model. Performance of the model is measured as the percentage of correctly predicted spikes in the final part of the stimulation. A threshold model generates spikes (brown) whenever the simulated voltage (light green) hits a dynamic threshold (dashed line).

The potential value of the competition is best illustrated for the *in vivo* data set, which allowed a reevaluation of previously published data from a neuron in the lateral geniculate nucleus of the brain (9). The winning submission (a threshold model) predicted the timing of 90.5% of the spike activity of this neuron (knowing its input, which was caused by visual stimulation of the retina), and thereby surpassed the performance of the previous data analysis by an astonishing 11%.

How well did the detailed biophysical neuron models perform? We don't know, because no prediction based on a detailed model was submitted. The reason may be that it is simply too difficult to tune the parameters of a detailed neuron model to perfection. However, systematic methods for automatic parameter tuning are just becoming available (10, 11).

Among the lessons to be learned from the INCF competition is that every neuron is different and one should not think of "the" model of a pyramidal cell or interneuron. Rather, parameters need to be tuned on a neuron-by-neuron basis. Another lesson is that the quality of a neuron model has to be measured on new data that are not accessible during the phase of parameter tuning. These new data (test set) can be statistically of the same type, but must be different from the data in the training set. In addition, making data publicly available is most rewarding if the data set is combined with a well-formulated task. A final lesson is that, for

tasks consisting of predicting spike activity times under single- or double-electrode current injection, simple neuron models of the threshold type that are augmented by adaptation (to describe neuronal fatigue) are sufficient in that they can predict all the predictable spikes. The good performance of threshold models is excellent news for studying properties of neural coding or dynamics of large neuronal networks using adaptive integrate-and-fire neurons. Stochastic versions of such threshold models, also called generalized linear models, have recently been successfully used to decode neural information in sensory (12) and motor (13) areas.

Threshold models give a phenomenological description of neural behavior, but provide only a weak link to the underlying biophysical causes of electrical activity. By construction, threshold models are rather limited in predicting the precise time course of the voltage during and after a spike, and cannot predict the influence of temperature dependence, changes in the chemical environment, or pharmacological manipulations of ion channels, whereas biophysical models of the Hodgkin-Huxley type can do all this. It may soon be possible to measure most parameters of biophysical models in a systematic fashion with a suitable combination of immunostaining methods to determine ion-channel distribution, calibrated measurements of ion-channel kinetics, and expression studies to identify tens of ion channels in individual cells. Automatic model construction along these lines is on

its way (14). Moreover, intricate nonlinear spatiotemporal effects on the dendritic tree such as the interplay of back-propagating action potentials (those that travel into a dendrite) with shunting inhibition, or local spikes in the concentration of intracellular calcium that are triggered by multiple, spatially distributed, synaptic inputs, are beyond the scope of threshold models. Although these nonlinear spatiotemporal aspects were difficult to quantify with traditional experimental methods, new imaging techniques that measure the instantaneous voltage time course across the dendritic tree at high spatial resolution in combination with a controlled multisite stimulation (15)—either by glutamate uncaging (16) or optogenetic methods (17)—will open the door to an era of quantitatively predictive biophysical models.

Competitions and model comparisons are widespread in the community of machine learning, where new computer algorithms are tested on benchmark data under well-defined procedures. With a few exceptions, the idea of benchmarking neuron models on a publicly available set of data in a prediction task has not yet found its way into the standard repertoire of neuroscience, but the increased numbers of participants in the INCF competition compared to earlier years (up from 9 to 33 submissions) indicate a paradigm shift in that respect.

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RETROSPECTIVE

Norman Ernest Borlaug (1914–2009)

Christopher Dowsell

Norman Borlaug spent his youth on a small farm in Iowa, dreaming of becoming a high school science teacher and athletics coach. In the end, he spent his life working to keep food production ahead of population growth. His last spoken words on the day of his death (12 September 2009) were “take it to the farmer.” This was his credo for 65 years, and his research added hundreds of millions of additional tons of grain to world harvests.

After completing doctoral studies in plant pathology at the University of Minnesota in 1942, and wartime service as a microbiologist for the DuPont Company, Norm went to Mexico in 1944 as part of the first Rockefeller Foundation agricultural team. Within 12 years, his team improved seeds and agronomic practices, boosting Mexican wheat production fivefold. He also built a cadre of agricultural scientists to help farmers maintain self-sufficiency. He was the first in the field at sunrise and the last to leave at sunset. No job was too menial for him. “Impact on farmers’ fields, not learned publications,” Norm would tell his team, “is the measure by which we will judge the value of our work.”

For 62 years, Mexico was Norm’s home. Beginning in the 1960s, the Rockefeller staff in Mexico shifted their focus to other countries through the newly created International Maize and Wheat Improvement Center, which Norm served until his death. Their program was built around three pillars: practical research to address production problems facing farmers, free exchange of seeds and information, and developing local researchers. He trained hundreds of young researchers from the developing world, forging a network that would spur a “Green Revolution” in wheat production, one of the great milestones in the history of world agriculture.

At the age of 70, Norm began teaching international agriculture to graduate students and speaking to undergraduates at Texas A&M University. He admired the agricultural experiment stations and cooperative extension services of such U.S. land grant universities, and understood that advocacy was key to getting modern agricultural technology into use. With the General Foods

Company (and later, through a private investor), Norm created the World Food Prize in 1986, to honor those who contributed significantly to the quantity, quality, and availability of food. Award recipients have hailed from around the world, to Norm’s great satisfaction. He was also proud of The Borlaug Dialogue, where global food and agricultural issues are discussed, and the World Food Prize Youth Institute, created to nurture future leaders. Some interns, as he had hoped, are pursuing careers related to agricultural research and development.

In 1985, Japanese philanthropist Ryoichi Sasakawa proposed that they launch an agricultural extension program in one or two African countries, following the Asian “Green Revolution” strategy. Former U.S. President Jimmy Carter also joined, pledging to lead efforts to influence agricultural policy-makers. Since then, the Sasakawa–Global 2000 agricultural program has operated in 15 sub-Saharan countries. Although Norm witnessed the same euphoria among African farmers as he had seen in Latin America and Asia, the outcomes were not the same. Africa lacked the necessary infrastructure—roads, railroads, and irrigation systems—as well as the market institutions to deliver needed inputs such as seed and fertilizer. Nor were there farmer incentives from governments to encourage modernization. “The potential is there,” he came to realize, “but you can’t eat potential.” Norm realized he would not live to see the Green Revolution in Africa, but saw signs of growing investment in smallholder agriculture and was optimistic that a transformation was coming.

In the last decades of his life, Norm became increasingly fascinated with the new tools and products of biotechnology and considered genetic engineering a logical progression from conventional plant-breeding research. But he worried about the lack of public sector research investment to balance the work of the private sector.

When he was awarded the Nobel Peace Prize in Oslo in 1970, the Speaker of the Norwegian Parliament noted that Norm had been

The innovative efforts of a plant scientist revolutionized farming, changed national agricultural policies, and fed the developing world.



selected, not so much for his great research achievements, but because of his untiring efforts to implement agricultural innovations. In accepting the Nobel Prize, Norm noted “that the responsibilities of the prize were far greater than the honor itself.” Indeed

they were. For the next nearly four decades, he worked tirelessly as an advocate for farmers, agricultural science and technology, and foreign international assistance. Over the years, he spoke to more than 250,000 university and high school students, delivered addresses at 450 conferences and symposiums to more than 200,000 professionals, consulted with policy-makers in 60 countries, and gave more than 500 press interviews. He served as a foreign member of the Academies of Science

in 12 countries and as an adviser to four U.S. presidents. Heads of government, such as Ayub Khan of Pakistan, Indira Gandhi of India, Deng Xiaoping of China, Raúl Alfonsín of Argentina, and Meles Zenawi of Ethiopia, listened to his counsel and made major policy changes that greatly accelerated agricultural development in their nations.

In 2006, at 92 years of age, Norm was diagnosed with cancer. His illness came as a shock, as he had seldom been sick, and he fully expected to be cured. His last great cause was to mount an international response to a new wheat stem rust disease (Uganda 99) capable of wiping out the world’s commercial wheat crop. In March 2009, he returned to Ciudad Obregón, Mexico, where the initiative was launched, to hear reports on the first year’s progress. It was to be his last trip. More than 300 top wheat researchers from around the world came to the meeting, as much to pay homage to Norm as to present research findings. An unspoken pledge was made to contain this deadly wheat disease before it wreaks havoc on global wheat production. He returned to Dallas, spending his final days with children, grandchildren, and great-grandchildren. Norman Borlaug was agriculture’s greatest spokesperson and is credited with saving hundreds of millions of lives worldwide. Quite a legacy.

10.1126/science.1182211

Evolution and Revolution in Odor Detection

Richard Benton

Molecular neuroscientists have a tendency to seek evolutionarily conserved mechanisms underlying the construction and function of animal brains. This approach unarguably helps to define fundamental principles of neurobiology by integrating insights from diverse model nervous systems. However, while what is true of the brain of a mouse or worm may be relevant to our own, a focus on commonalities overlooks the fact that different animal nervous systems have evolved to operate in distinct ecological contexts. Nowhere is this truer than in the olfactory system, which underlies the detection of myriad volatile chemicals in the environment. Animal olfactory systems display enormous evolutionary capacity, as species acquire and discard olfactory receptor genes, neurons, and behaviors in an ever-changing landscape of external chemical stimuli. These modifications often reflect the fact that most relevant odors for a species are themselves derived from evolving organisms such as plant food sources, animal predators, and potential mates.

Insect olfactory systems have been important models since the observations of Fabre in the early 1900s of the potency of a volatile chemical released by a caged female emperor moth in attracting male suitors. Surprisingly, insects lagged well behind vertebrate models in molecular investigations, as odorant receptor (OR) genes were identified in the fruit fly, *Drosophila*, nearly a decade after the discovery of mammalian ORs. Nevertheless, the small number of *Drosophila* ORs (62, compared with >1000 in mice) permitted comprehensive functional analysis of the role of these receptors and their circuits in odor perception (1, 2). Many common properties of insect and vertebrate olfactory systems have been appreciated, notably, (i) that individual olfactory sensory neurons (OSNs) express just one type of OR, (ii) that the axons of OSNs expressing the same receptor converge to a



Eppendorf and *Science* are pleased to present the prize-winning essay by Richard Benton, the 2009 winner of the Eppendorf and *Science* Prize for Neurobiology.

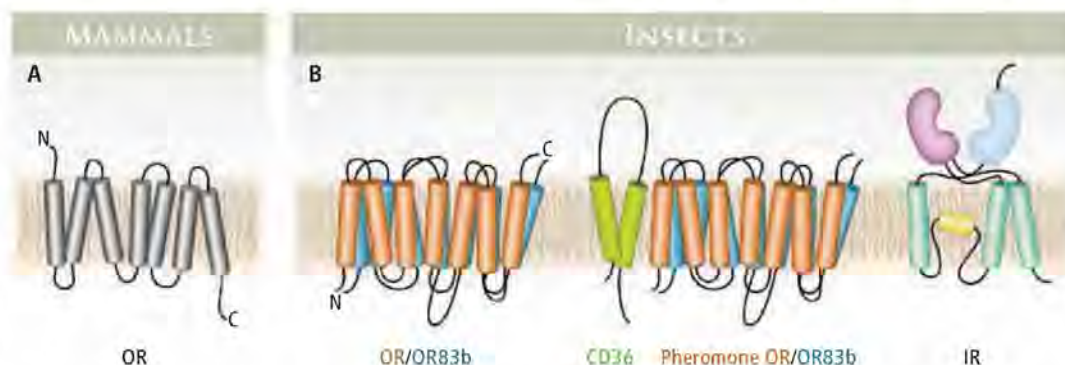
defined glomerulus within the primary olfactory center, and (iii) that odors are recognized by specific combinations of ORs to create a spatial "code" of glomerular activation (3).

Justified by this apparent conservation in olfactory system organization across 500 million years of evolution, my research, with collaborators at The Rockefeller University, aimed to use *Drosophila* to investigate the mechanisms by which ORs transform odor recognition into neuronal activity. This was a goal, however, that rapidly shifted focus. When initially identified, *Drosophila* OR proteins were predicted to contain seven transmembrane domains—a structural hallmark of G protein-coupled receptors (GPCRs)—leading to the widespread belief that insect ORs, like their mammalian counterparts, signal via G protein-mediated second-messenger

Similarities and differences between phyla give insights into the evolution of the olfactory system.

cascades. However, upon bioinformatic reexamination of insect OR sequences, we noted that these receptors exhibited no significant similarity to vertebrate ORs or other GPCRs and, unexpectedly, were predicted to have an inverted membrane topology. We confirmed this provocative computational prediction experimentally, using antibodies against OR epitopes to map their location relative to OSN ciliary membranes. These findings led us to conclude that the long-held dogma of insect ORs being GPCRs was incorrect; rather, they defined a distinct family of transmembrane proteins (4–6). How insect ORs work remains contentious, although recent analysis by others suggests that they function as odor-gated ion channels (7, 8).

At this point, we were faced with a problem: using *Drosophila* as a "model" system, but working on receptors that were unique to insects. We decided to fly in the face of this insect-specificity by designing a bioinformatics screen to identify additional factors acting in peripheral odor detection, selecting genes that displayed the same insect-specific orthology as the ORs (by comparative analysis of *Drosophila*, mosquito, and noninsect genomes) and OSN-specific expression (by expressed sequence tag analysis). These simple criteria were highly effective in recovering all known classes of insect olfactory genes



Molecular mechanisms of odor detection in mammals and insects. (A) Mammalian ORs and other classes of vertebrate chemosensory receptors are members of the seven-transmembrane domain GPCR superfamily, with extracellular N termini and intracellular C termini. (B) (left) By contrast, insect ORs define a distinct family of seven-transmembrane domain receptors with inverse topology to GPCRs and function as heteromeric complexes of an odor-binding OR (red) and a co-receptor OR83b (blue) that likely form an odor-gated ion channel. (Middle) Insect ORs responsible for detecting lipid-derived pheromones additionally require the function of a CD36 protein, a lipid-binding cell surface receptor that acts in immune recognition in other organisms. (Right) A second family of insect olfactory receptors are the IRs, which are homologous to the ionotropic glutamate receptor family of ligand-gated ion channels.

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and many uncharacterized molecules (9).

Of the latter group, we first focused on a transmembrane protein, SNMP, that is distantly related to the mammalian lipid-binding protein CD36. We showed that SNMP is expressed specifically in pheromone-sensing OSNs and localizes with ORs in the sensory cilia. Electrophysiological analysis of *SNMP* mutant OSNs demonstrated that it is essential for pheromone-evoked neuronal activity. Most insect pheromones are lipid-derived, leading us to propose that SNMP acts as a cofactor for pheromone-sensing ORs by directly capturing pheromone molecules on the surface of OSN cilia (9, 10). Intriguingly, other CD36-related proteins function as cofactors for Toll-like receptors, permitting detection of bacterially derived lipids to initiate innate immune signaling. Our results therefore highlight a mechanistic parallel between conspecific recognition through pheromonal communication and pathogen recognition through the innate immune system. Molecular homologies have also been noted in pheromone and immune detection in mammals (11); a future challenge is to understand the evolutionary basis of such connections.

Our earlier investigations of ORs also revealed a second problem: that flies lacking the function of all receptors (owing to mutation of the OR co-receptor OR83b) still exhibited behavioral responses to many odors. This obser-

vation implied the existence of additional odor-detection mechanisms. Our bioinformatics screen identified several uncharacterized ionotropic glutamate receptors (iGluRs)—ligand-gated ion channels best studied for their roles in synaptic communication—but these receptors were unusual, bearing divergent ligand-binding domains that lack glutamate-interacting residues. Three lines of evidence supported our hypothesis that these proteins, named ionotropic receptors (IRs), represent a new family of olfactory receptors: First, IRs are expressed in specific combinations in OSNs that do not express ORs; second, the proteins localize to sensory cilia rather than synapses; and finally, misexpression of an IR in an ectopic neuron is sufficient to confer novel odor responses. IRs are therefore likely to define a previously unappreciated “second nose” in insects (12). By contrast to insect ORs, iGluR/IR-like proteins are present across animals, plants, and prokaryotes, which hints that these receptors may represent an ancient mechanism for sensing both intercellular and external chemical cues.

Our studies of the biology of *Drosophila* odor detection have revealed molecular surprises that invite reconsideration of the basis of the striking similarities in olfactory system organization and function across species (13). Was there a primitive olfactory system in the common ancestor of insects and vertebrates,

in which subsequent drastic divergence of the odor-detecting receptors was uncoupled from the maintenance of neuroanatomical and physiological logic? Or, does the common design of olfactory systems across different phyla reflect convergent evolution, indicative of the essential properties of a sensory system responsible for detecting innumerable chemical stimuli? Distinguishing these possibilities is not trivial, but either would yield insight into the mechanisms by which at least this part of the nervous system arose and evolved.

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10.1126/science.1181998

2009 Grand Prize Winner



The author of the prize-winning essay, **Richard Benton**, obtained his Ph.D. in 2003 for research on the molecular basis of cell polarization performed in the group of Daniel St. Johnston at The Wellcome Trust/Cancer Research U.K. Gurdon Institute at the University of Cambridge. For his postdoctoral training, he joined Leslie Vosshall's laboratory at The Rockefeller University, New York, where he became interested in olfactory signaling mechanisms in insects. During his postdoc he was supported by fellowships

from the European Molecular Biology Organization and the Helen Hay Whitney Foundation. He established his laboratory as assistant professor at the Center for Integrative Genomics at the University of Lausanne, Switzerland, in September 2007. In 2008, he was awarded a European Research Council Starting Independent Researcher Grant. His group studies the genetic, neural, and evolutionary basis of chemosensation in the fruit fly, *Drosophila*.

Finalists

Max Heiman, for his essay “The Brain That Nature Built.” Dr Heiman learned how to pipette in 1995 as a summer student with Steve Reeves at Massachusetts General Hospital; while there, he wrote Webcutter, one of the earliest online DNA analysis programs.



He received a B.S. in Biology in 1997 from Yale University, working with Frank Ruddle on homeobox genes in mouse development. As a graduate student with Peter Walter at the University of California, San Francisco, he identified the first protein implicated in membrane fusion in yeast mating, a process analogous to sperm-egg fusion, and received his Ph.D. in Biochemistry in 2002. He has been a fellow of the Howard Hughes Medical Institute and the Jane Coffin Childs Memorial Fund. Since 2003, he has conducted postdoctoral work with Shai Shaham at The Rockefeller University, using *Caenorhabditis elegans* to study the assembly of neuronal shapes.

David McLean, for his essay “Shifting Gears to Change Speeds in the Spinal Cord.” Dr. McLean was born in Perth, Scotland, but grew up in Canton, New York, USA. He returned to Scotland to study at the University of St. Andrews, where he received his B.Sc. in Biology in 1997 and then his Ph.D. in Neurobiology in 2001 with Keith Sillar. In 2002, he joined Joseph Fetcho's laboratory as a postdoctoral fellow, where he studied the spinal control of locomotor movements.

He is now at the Department of Neurobiology and Physiology at Northwestern University, where he continues to pursue his interest in the development and plasticity of motor networks.



For the full text of finalist essays and for information about applying for next year's awards, see *Science* Online at www.sciencemag.org/feature/data/prizes/ependorff/.



INTRODUCTION

So You Want to Learn How to Network?

EVER SINCE HUMAN BEINGS BEGAN TO SYSTEMATICALLY INVESTIGATE THE NATURAL world, the introduction of new techniques has pushed the boundaries of our knowledge and revolutionized the way in which we see ourselves and our place in the cosmos. Just think of Galileo and the telescope or Golgi and silver staining for nervous tissue.

With the development and introduction of new techniques coming faster than ever, we highlight in this year's neuroscience special issue some of the recent advances that have shown to be particularly promising. We invited leading neuroscientists, each of whom investigates a different organizational level of the nervous system, to give an overview of the latest methodological developments in their area of interest, to critically analyze the potential and limitations of the available techniques, and to present their vision of what the future might hold in store.

How and why are specific cells within a neural circuit, and not their neighbors, recruited during learning? Silva *et al.* (p. 391) review new molecular and cellular methods that allow the tagging and direct study of neurons *in vivo*, as well as techniques for the activation and inactivation of these neurons in behaving animals. These approaches may have the potential to revolutionize studies of the function of brain circuits, including memory allocation. The technique of optogenetics has been around for less than a decade but, as reviewed by Miesenböck (p. 395), promises to be one of the major developments in the field of systems neuroscience. It will undoubtedly provide novel insights into the organization of neural circuits and the regulation of their interactions. Functional neuroimaging is now without doubt the predominant technique for looking at the brain as a whole. Friston (p. 399) reviews its enormous achievements as well, summarizing the ongoing debates in the field. In a related Perspective, Gerstner and Naud (p. 379) look at the latest advances in computational neuroscience and show how improvements in our approaches to neuronal modeling can help us make more accurate and testable predictions about the behavior of neurons.

In a Q&A, News writer Greg Miller talks to physicist Mark Schnitzer about the future of neuroscience microscopy, particularly his pioneering high-throughput efforts. And Miller also explores whether the Alzheimer's Disease Neuroimaging Initiative has succeeded at developing better ways of tracking the condition and testing drugs for it.

— PETER STERN

Neuroscience Methods

CONTENTS

News

- 386 Alzheimer's Biomarker Initiative Hits Its Stride
Longitudinal Alzheimer's Studies Go Global
- 390 Massively Parallel Brain Imaging

Reviews

- 391 Molecular and Cellular Approaches to Memory Allocation in Neural Circuits
A. J. Silva et al.
- 395 The Optogenetic Catechism
G. Miesenböck
- 399 Modalities, Modes, and Models in Functional Neuroimaging
K. J. Friston

See also Editorial p. 339 and Perspective p. 379

Science

NEWS

Alzheimer's Biomarker Initiative Hits Its Stride

An effort to develop biomarkers for Alzheimer's disease is churning out new data and making plans to expand



IMAGINE YOU'RE AN EXECUTIVE AT A PHARMACEUTICAL company and your scientists have just briefed you on a promising new drug for Alzheimer's disease. Dollar signs are no doubt swirling before your eyes. Alzheimer's afflicts 4 million people in the United States alone, and burgeoning elderly populations in countries like India and China portend skyrocketing demand for decades to come. But hold on. First, you have to run clinical trials. Because the cognitive tests and clinical measures used to gauge the efficacy of Alzheimer's treatments are notoriously variable, you may need to enroll 1000 people or more in the hope of seeing a statistically significant benefit. And given that Alzheimer's can be diagnosed definitively only by examining the brain after death, you can expect that at least 10% of the subjects won't have Alzheimer's but some other type of dementia that won't respond to your drug. Even those who do have Alzheimer's may be too far gone to benefit. Suddenly, those dollar signs begin fading from view.

Such concerns are what motivated pharmaceutical companies to band together and join the National Institutes of Health to form an innovative partnership, the Alzheimer's Disease Neuroimaging Initiative (ADNI). Launched in October 2004 with \$64 million to fund its first 5 years, its *raison d'être* is to

develop methods for improving Alzheimer's disease clinical trials. ADNI has enrolled more than 800 volunteers between the ages of 55 and 90—roughly a quarter of them healthy, another quarter clinically diagnosed with probable Alzheimer's, and the rest diagnosed with mild cognitive impairment (MCI), a condition that often presages Alzheimer's. They undergo testing every 6 to 12 months with a variety of methods, including magnetic resonance imaging (MRI), positron emission tomography (PET) brain scans, and lumbar punctures to collect cerebrospinal fluid (CSF). The hope is to find biomarkers—signs of brain atrophy in an MRI scan, for example—that track the progression of Alzheimer's more faithfully than do the cognitive and clinical measures now used in treatment trials.

Five years in, a trickle of data is becoming a torrent. ADNI researchers are now poring over brain scans and other biomarker data to document changes as people who started out with a clean slate of cognitive health have developed MCI, and as those with MCI have progressed to Alzheimer's. ADNI's organizers caution that it's too early to draw definitive conclusions, but some potentially useful lessons are emerging, including hints about which biomarkers best

Into the scanner. Researchers hope to use MRI brain scans to track the progression of Alzheimer's disease.

track different stages of the disease. Pharmaceutical companies are already incorporating some of the ADNI measures into clinical trials, and researchers in Europe, Asia, and Australia are developing similar initiatives (see box, p. 388).

"As far as I can see, it's just been a phenomenal success," says neurologist Michael Weiner of the University of California (UC), San Francisco, and the Veterans Administration Medical Center. Weiner, who is ADNI principal investigator, and the other project leaders found out last month that they'd won a \$24 million Grand Opportunities grant from the National Institute on Aging that will enable them to expand the study to include people with even earlier stages of MCI. And Weiner says a proposal for ADNI2, a continuation of ADNI that would enroll hundreds more volunteers and expand the genetic testing arm of the study, will be sent off by the end of this month.

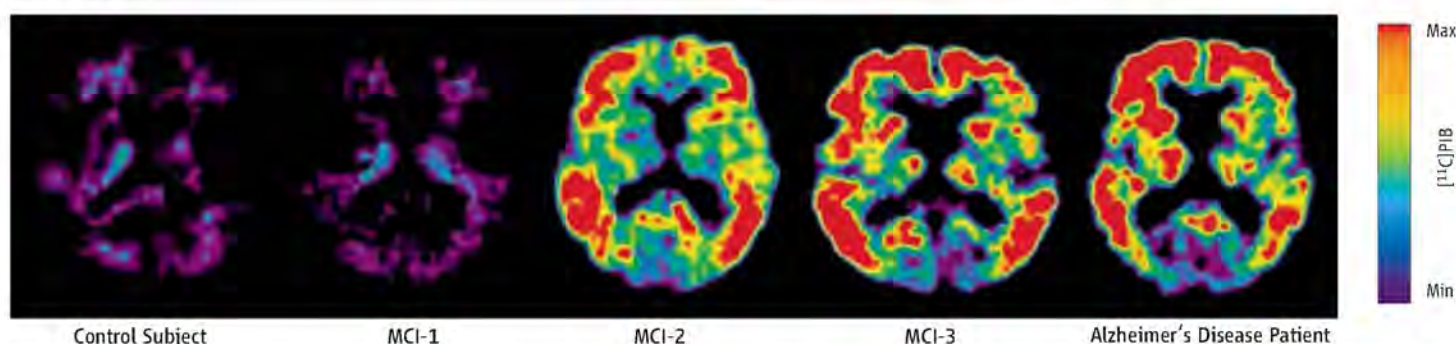
Tracking a killer

Although several drugs provide modest improvements in the memory impairments and other cognitive problems caused by Alzheimer's, so far no treatment has been proven to slow the underlying neurodegeneration (*Science*, 29 July 2005, p. 731). A handful of promising candidates have flopped in recent clinical trials, sparking much discussion about whether the field's longstanding leading hypothesis—that the accumulation of the β -amyloid peptide and formation of amyloid plaques is the core mechanism of Alzheimer's disease—is in need of revision, or worse.

Yet many researchers contend that the recent trials are less a death knell for the amyloid hypothesis than an illustration of the need for better biomarkers. For one thing, they point out, the trials all enrolled people who already had a clinical diagnosis of Alzheimer's disease. For these patients, who presumably have significant neurodegeneration, clearing β -amyloid from the brain may simply be too little too late. The real test for the amyloid hypothesis will come from identifying people earlier in the course of the disease and giving them anti-amyloid treatments to see if it delays or prevents dementia. "It's now quietly recognized that Alzheimer's disease pathology is probably present 10 or 20 years before someone becomes demented," says Weiner.

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PIB SCANS



Not a pretty picture. Amyloid deposits make for a colorful PIB scan of an Alzheimer's patient's brain (*far right*). People with MCI show diverse results.

Although the original goal of ADNI was to streamline clinical trials by developing biomarkers that track the progression of the disease more reliably than conventional clinical and neuropsychological measures do, Weiner says it's becoming apparent that some of the biomarkers under investigation may be valuable for diagnosing or even predicting Alzheimer's in patients suffering from only mild memory loss.

The three types of biomarker measurements included in the original plans for ADNI—anatomical MRI scans, PET scans to measure metabolic activity, and CSF samples—were chosen because they'd already shown promise in mostly smaller, single-center studies. ADNI researchers developed extensive protocols so that data collected at the 59 centers could be combined and compared. Standardizing complex procedures such as MRI and PET scanning across multiple centers with different equipment wasn't easy, says neurologist William Jagust of UC Berkeley, who heads ADNI's PET core. "If you'd told me 10 years ago I'd be heading a multicenter imaging study, I'd have thought you were crazy," he says. "The technology is just so complicated."

For the neuroimaging community, ADNI is also an unusual experiment in open-access science. All of the brain scans and other data procured so far are freely available to the scientific community at the ADNI Web site (www.loni.ucla.edu/ADNI/), typically within about a week of being collected, Weiner says. "Hundreds of scientists all over the world have made many tens of thousands of downloads," he says. A significant number of the several dozen papers published so far

on ADNI data have been authored by researchers not directly funded by the project, he notes. The open-access policy has given ADNI researchers an incentive to analyze and publish their data quickly, Weiner says. "It's really made me realize the power of releasing all the data."

A late addition

Just as ADNI was getting under way in 2004, researchers at the University of Pittsburgh published the first report on a new method for detecting β -amyloid in the living human

Several larger studies that got under way before ADNI have found that about 30% to 35% of healthy people in their 70s and 80s are PIB+, says Chet Mathis, who was part of the team that developed PIB and now heads the β -amyloid imaging core of ADNI. (Mathis has a financial interest in PIB via a licensing agreement with GE Healthcare.) To some researchers, the PIB findings, along with earlier postmortem studies that reported amyloid plaques in the brains of elderly people who died without suffering dementia, are another strike against the amyloid hypothesis, Mathis says. But it's also possible that the healthy PIB+ individuals will eventually develop the disease, Mathis notes. He and others say following those individuals—in ADNI and other longitudinal studies—will be an important test of the amyloid hypothesis.

Indeed, three non-ADNI studies published since 2008 suggest that PIB may be useful for identifying which MCI patients are most

likely to progress to Alzheimer's, Mathis says. Pooled together, those three studies found that nearly 60% of PIB+ MCI patients advanced to Alzheimer's disease within a year or two, compared with less than 5% of the PIB-MCI patients. The ADNI data show a similar but less pronounced trend, Mathis says. All in all, he says, the findings so far suggest that PIB is "among the earliest biomarkers" for picking up signs of Alzheimer's.



This is spinal tap. Cerebrospinal fluid may contain clues about one's risk for Alzheimer's disease.

brain with a PET scan. The method uses a radioactive compound, called the Pittsburgh Compound-B (PIB), that binds to amyloid plaques. Researchers scrambled to secure funding to add on a PIB component to the original ADNI grant and test it in about 100 ADNI volunteers. In what came as a surprise to some researchers, nine of the 19 volunteers from the healthy control group tested "PIB+," indicating significant β -amyloid deposits in the brain.

Compounds in spinal fluid may also indicate early signs of trouble, says Leslie Shaw of the University of Pennsylvania, who co-directs ADNI's CSF biomarker core. Previous studies found that the CSF of Alzheimer's patients contains low levels of β -amyloid (possibly because circulating levels of the peptide decrease as it becomes bound up in plaques) and high levels of tau, the protein that makes up the fibrillary tangles that are another pathological hallmark of the disease. In an April paper in the *Annals of Neurology*, Shaw and colleagues reported that the same pattern—low β -amyloid, high tau—was present in roughly 90% of CSF samples from ADNI volunteers who entered the study with MCI and progressed to Alzheimer's within the 1st year.

A major theme emerging from the ADNI data is that different biomarkers may prove more useful for evaluating people at different points on the continuum from normal aging to mild cognitive impairment to Alzheimer's. Although CSF and PIB may be useful for predicting who is most likely to progress from MCI to Alzheimer's, other biomarkers may be more sensitive to changes at later states of the disease, says Clifford Jack Jr. of the Mayo Clinic in Rochester, Minnesota, who heads ADNI's MRI core. In a recent analysis, Jack and colleagues found that an MRI measurement of brain atrophy correlated well with cognitive changes over the course of a year. PIB measurements, on the other hand, changed little during this time span, the team reported in the May issue of *Brain*. In that study, Jack and colleagues used a "boundary shift integral" technique developed by Nick Fox of University College London to track changes in the fluid-filled ventricles in the brain, which expand as brain tissue degenerates. This algorithm is just one of many ways to quantify changes in MRI scans, and Jack notes that other ADNI researchers have demonstrated similarly promising results with other methods (see MRI image, p. 389).

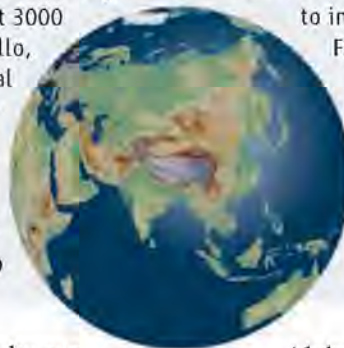
The other major neuroimaging component of ADNI, fluorodeoxyglucose (FDG) PET scanning, also tracks cognitive changes, says Jagust. Unlike MRI, which produces images of brain anatomy, FDG-PET

Longitudinal Alzheimer's Studies Go Global

In the past few years, several international projects inspired in varying degrees by ADNI (see main text) have gotten off the ground. In Japan, neurologist Takeshi Iwatsubo of the University of Tokyo leads a national Alzheimer's neuroimaging study very similar to ADNI. In China, where the population over 60 is expected to triple by 2050, three large-scale longitudinal studies are under way; each plans to enroll at least 3000 people, says Maria Carrillo, who helps coordinate global Alzheimer's studies as senior director of medical and scientific relations for the Alzheimer's Association. Plans for a Europe-wide ADNI-like effort appear to

have fizzled for political and logistical reasons, but several countries have their own projects, many of which have adopted ADNI methods, says Giovanni Frisoni of Fatebenefratelli Hospital in Brescia, Italy.

The Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing (AIBL) will examine the role of lifestyle factors such as diet and exercise in cognitive aging, in addition to investigating blood biomarkers, FDG-PET, MRI, and PIB. The study is not modeled on ADNI, but researchers have adopted some of its methods so that all the neuroimaging data will be compatible, says AIBL Director David Ames of the University of Melbourne. —G.M.



uses radioactively tagged glucose to measure brain metabolism. In people with Alzheimer's, FDG-PET reveals reduced metabolism in the brain, particularly in regions of the parietal and temporal lobes, Jagust says. "FDG-PET is probably going to turn out to be a pretty good predictor in the early-to-middle stages of the disease," he says. One popular hypothesis is that the method will fill in the gap between β -amyloid biomarkers and MRI, picking up changes in brain metabolism that occur when β -amyloid deposition is under way but not yet advanced enough to cause structural damage that shows up on an MRI scan.

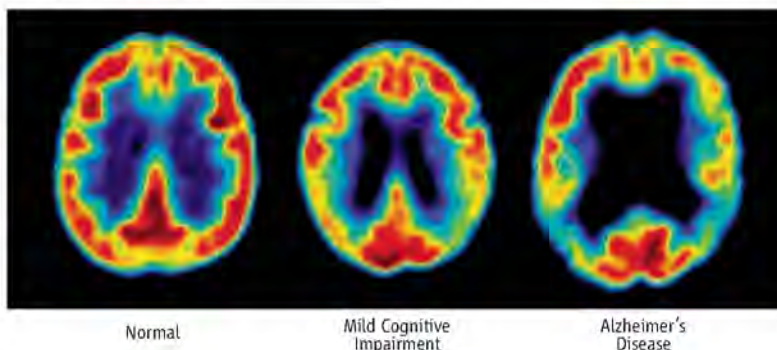
Methods on trial

But will ADNI pay off for the pharma companies paying roughly a third of its budget? Their road to regulatory approval for

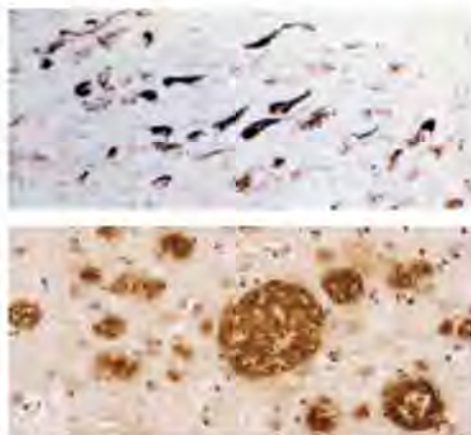
Alzheimer's disease treatments is difficult because the only measures now accepted by the Food and Drug Administration (FDA) for evaluating treatments are cognitive and clinical scales. But these measurements have their downsides, says Laurel Beckett of UC Davis, who leads ADNI's biostatistics core. "The cognitive measures are just so noisy," Beckett says. "They go up and down for who knows what reasons because people just have good days and bad days." That variability, coupled with the slow progression of the disease and the relatively modest effects of most candidate treatments, is why Alzheimer's trials have had to enroll so many patients in hopes of demonstrating a statistically significant effect.

Some researchers hope it will one day be possible to use surrogate markers in clinical trials for Alzheimer's therapies, much as measurements of blood pressure and cholesterol levels have been used to approve drugs for heart disease. Several analyses of the ADNI data have suggested that some biomarkers, such as changes in brain volume measured by MRI, could reduce the number of patients needed by an order of magnitude. However, any Alzheimer's disease biomarker must not only track the progression of the disease accurately but

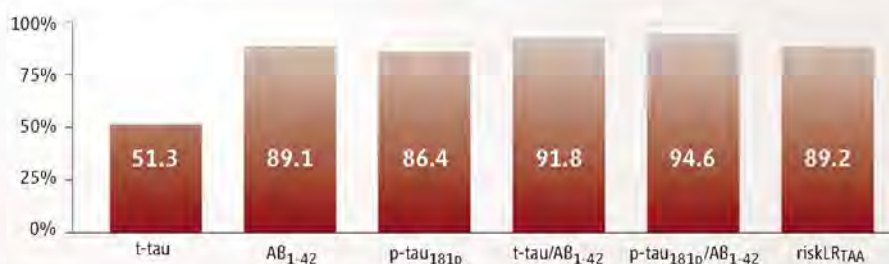
FDG-PET SCANS



Energy crisis. FDG-PET scans show reduced metabolic activity (warm colors) in the brains of people with Alzheimer's disease (far right).



CSF BIOMARKERS



Predicting trouble. A high percentage of ADNI-MCI subjects who progressed to Alzheimer's disease had abnormal CSF levels of various versions of tau (*top left*) and β -amyloid (*bottom left*), two biomolecules thought to play a role in Alzheimer's pathology.

also respond to treatment in a way that mirrors actual clinical improvements, says Russell Katz, the director of the division of neuropharmacological drug products for FDA. It's an exciting possibility, Katz says, but "we're nowhere near there."

Even so, companies are already incorporating ADNI-vetted biomarkers. "Every company that's working in AD [Alzheimer's disease] drug development is designing trials based on ADNI data right now, not as the only tool but as a significant tool," says neurologist Paul Aisen of UC San Diego, who co-chair's ADNI's clinical core and oversees government-sponsored clinical trials as director of the Alzheimer's Disease Cooperative Study.

At least one company is already using CSF biomarkers to screen subjects for a clinical trial, and others are considering it, says Aisen. Including only those people who show both β -amyloid aberrations and memory problems may help weed out misdiagnosed Alzheimer's cases and provide a better test of the proposed therapy.

Some companies anticipate biomarkers will help establish that their treatments strike at the roots of the disease. Eli Lilly, which has two compounds in phase III trials for Alzheimer's, is using several biomarkers—including MRI, FDG-PET, and β -amyloid CSF and PET—in hope of demonstrating that these treatments provide biological as well as clinical benefits. "Our studies are set up so that they look quite a bit like ADNI," says Eric Siemers, the medical director of Lilly's Alzheimer's team.

Such evidence won't directly influence

the decision to approve the drug. But demonstrating a positive change in a biomarker—in addition to establishing a clinical benefit—might earn a company the right to claim its drug slows the decline of Alzheimer's disease, something no drug currently on the market can claim. Says Katz: "You can imagine the marketing advantage to the first company that gets a drug whose label says it's approved to slow the progression of Alzheimer's disease."

within each group there is considerable diversity in the baseline biomarker profiles of different individuals and in the changes that have occurred during the first 2 years of the study. And that diversity has predictive value, Beckett says. "We are definitely seeing subtle changes in biomarkers that foreshadow both the cognitive outcomes and brain changes."

And the data will continue to roll in. Plans for ADNI2 include a greatly expanded

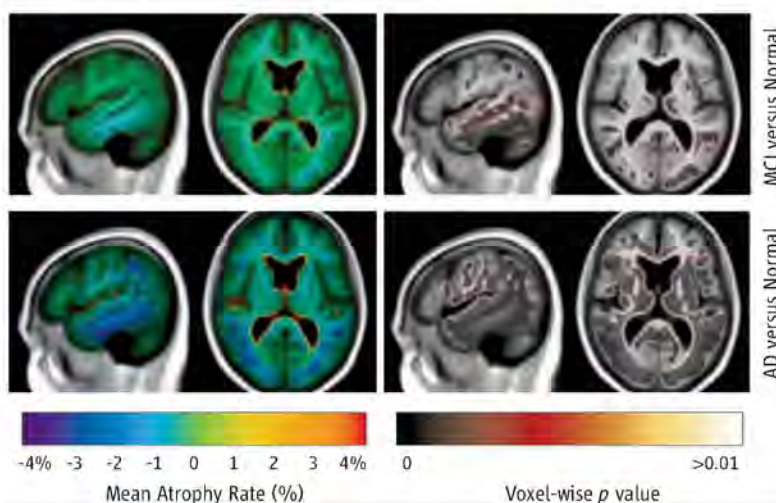
β -amyloid-imaging arm. Because PIB is based on the short-lived Carbon-11 isotope, it has to be made on site, which limited its use to the 14 ADNI centers with their own cyclotron. If approved, ADNI2 will use a newer PET ligand based on Fluorine-18, which has a longer half-life, allowing it to be shipped to centers that can't make it themselves.

ADNI2 would also include a beefed up genetics component, which, like PIB, was a late add-on to the first ADNI study. Researchers published the first work from this effort, a genome-wide association study in ADNI subjects, in August in *PLoS ONE*, reporting several

genetic variations linked to hippocampal atrophy in Alzheimer's patients. Several more studies are nearing publication and many more are just waiting to be done, says the head of ADNI's genetics core, Andrew Saykin of Indiana University in Bloomington. "Investigators around the world are going to be chewing on this very rich data for a very long time."

—GREG MILLER

MRI SCANS



Atrophy analysis. Paul Thompson and colleagues at the University of California, Los Angeles, have developed a tensor-based morphometry technique to measure brain atrophy in the brains of MCI and Alzheimer's patients.

Different trajectories

The 2-year followup data for ADNI subjects has just come in, and Beckett says she's been "going mad trying to compile the data and bring some order out of the chaos." She says what's most striking about the analysis so far is that although the individuals in the normal, MCI, and Alzheimer's groups were intentionally chosen to be homogenous in their clinical profiles,

Massively Parallel Brain Imaging

Mark Schnitzer is building tools to visualize neural activity in the fruit fly brain—100 flies at a time

INVENTING TECHNOLOGY THAT PUSHES THE frontiers of brain research is the passion of applied physicist and neuroscientist Mark Schnitzer. His lab at Stanford University in Palo Alto, California, has cranked out several cutting-edge devices in recent years, including a 1.1-gram fluorescence microscope that can be mounted on a freely moving mouse to monitor the activity of neurons and glial cells and a 2.9-gram fiber-optic two-photon microscope for imaging cells deep inside the brain of an active rodent.

Now he's working on an even bolder project, developing optical imaging technology for simultaneously recording neural activity in the brains of 100 fruit flies (*Drosophila melanogaster*), thanks to a \$1.3 million grant from the Keck Foundation and a \$2.5 million Director's Pioneer Award from the National Institutes of Health. Compared with current methods, which allow imaging only one fly at a time, such "massively parallel brain imaging" could open up research questions that have been out of reach, Schnitzer says. His comments have been edited for brevity. —GREG MILLER

Q: What was the impetus for this project?

M.S.: The basic idea was that neuroscientists have been starved for data, particularly brain-imaging data in awake, behaving animals. With invertebrates, and in particular with genetic model species like *Drosophila*, there's an opportunity to achieve a greater level of throughput and automation and at the same time to look at activity in populations of individual neurons as an animal executes a behavior.

Q: How do you hope to accomplish that?

M.S.: It's very early days, but our goal is to be able to examine 100 fruit flies in parallel. [The brains of individual] fruit flies have been examined previously by two-photon imaging with genetically encoded sensors for intracellular calcium, which is often used as an indicator of excitation in neurons. One of the limiting steps in this is the need to manually dissect the cuticle of the fly to access the brain optically. The first part of achieving higher throughput is to automate this step, so we're developing computer-directed laser microdissection.

The basic idea would be to have a tray of flies, each fixed in place, and have the laser do the cutting automatically.

With two-photon fluorescence microscopy, one can record in real time the calcium dynamics of neurons that have been genetically labeled with a calcium indicator. With a grid of laser beams, you could do that with an array of flies and collect multiple streams of data, each coming from the brain of an individual animal.



A brainy idea. Mark Schnitzer wants to develop high-throughput methods for optical imaging in fruit flies (shown here in an early prototype).

Q: Given that the flies have to be fixed in place, won't that limit the range of possible experiments?

M.S.: Flies can exhibit motor behavior while fixed in place by walking on a stationary ball that rotates under their feet. That paradigm has been used for decades and was a point of inspiration for us. They can also respond to both odors and visual stimuli.

Q: Is the goal here just to collect data more quickly?

M.S.: Speed is certainly important. But I think if it really is possible to look at 100 flies in parallel, it will open up questions that can't be addressed today because the experiments are simply too prohibitive. One class of experiment would be to examine many flies of the same genetic strain and try to understand any differences in brain function, to look for elements of individuality among animals with the same genome. A complementary type of experiment would be to look at many flies that each have a somewhat different genetic makeup and try to understand how these flies behave and process information differently.

Q: Do you envision this as something an individual lab would have, or would it be a shared resource?

M.S.: It depends on a number of things, including how successful the technology is and how much it costs. If it turns out to be a large, pricey device, or if it's used in combination with other methods, that might justify a centralized location. For example, other groups are working on techniques for mapping all of the connections in neural circuits and on ways to automate tissue processing and histology. Some of these tools might get packaged together. One might first examine a large number of flies when they're alive and then subsequently look at them as histological specimens.

Q: Going forward, how do you think such methods might change the way neuroscience is done?

M.S.: High-throughput experimentation allows you to be much more systematic. I think we'll see a trend towards trying out all, or at least a large number, of the logical possibilities, as opposed to testing one hypothesis at a time. And I think as large data sets develop, the field will necessarily become more collaborative. It will require specialist skills in data mining and statistical and computational tools. We're already seeing a lot of that.

Q: What motivates you to build microscopes?

M.S.: The challenge of being able to watch the underlying cellular components that give rise to behavior, the challenge of seeing macroscopic phenomenon come to life out of microscopic components.

REVIEW

Molecular and Cellular Approaches to Memory Allocation in Neural Circuits

Alcino J. Silva,* Yu Zhou,† Thomas Rogerson, Justin Shobe, J. Balaji

Although memory allocation is a subject of active research in computer science, little is known about how the brain allocates information within neural circuits. There is an extensive literature on how specific types of memory engage different parts of the brain, and how neurons in these regions process and store information. Until recently, however, the mechanisms that determine how specific cells and synapses within a neural circuit (and not their neighbors) are recruited during learning have received little attention. Recent findings suggest that memory allocation is not random, but rather specific mechanisms regulate where information is stored within a neural circuit. New methods that allow tagging, imaging, activation, and inactivation of neurons in behaving animals promise to revolutionize studies of brain circuits, including memory allocation. Results from these studies are likely to have a considerable impact on computer science, as well as on the understanding of memory and its disorders.

How and where specific items are stored has a lot to do with how easily they can be retrieved and used. Organized storage saves space (for example, superfluous or duplicate items can be eliminated), minimizes search times, and reduces errors during retrieval. Memory allocation refers to a set of processes that determine where information is specifically stored in a neural circuit. Are there neurobiological processes that determine which cells and synapses within a given circuit are engaged during learning, or is this random? Does memory allocation involve competition between different cells (or synapses) activated during learning? Do memory allocation processes take place at different time scales? The allocation of information is an especially important problem for the brain because of the enormous number of related memories stored throughout a lifetime. Without a mechanism that appropriately groups and separates memories, how would the brain store so many complex memories of both similar and discrete events? One possibility is that the brain stores related memories in overlapping populations of neurons and in synapses within the same dendritic branch, so that activation of one component of the memory increases the likelihood of retrieval of other related components. This strategy would require mechanisms that regulate where memory is stored in neural networks.

The principles and mechanisms of memory allocation are not only fascinating because of the insights they provide into how the brain stores

information, but they also have far-reaching implications for the design of artificial intelligence systems and for cognitive disorders. The central question that we will address here is whether memory allocation is random or whether specific mechanisms determine where information is

stored within a neural network. Recent findings suggest that there are mechanisms that regulate the allocation of memory to specific neurons in a network and modulate the synaptic selection processes that determine where these memories are stored (Fig. 1).

CREB and Memory Allocation

The cyclic adenosine monophosphate (cAMP) responsive element-binding protein (CREB) regulates the transcription of other genes and has a well-known role in the stability of synaptic potentiation and memory (1, 2). A recent series of papers (3–5) provided compelling evidence that there are molecular and cellular processes that determine which cells are recruited to store information within a neural circuit. In particular, this work suggested the hypothesis that CREB activated during learning triggers changes in the cell (such as an increase in excitability) that then affect whether that cell participates in subsequent memories. This idea was tested by artificially increasing the levels of CREB in amygdala neurons with the use of a replication-defective herpes viral vector. The initial results showed that higher CREB levels increase the probability (~threefold) that amygdala neurons participate

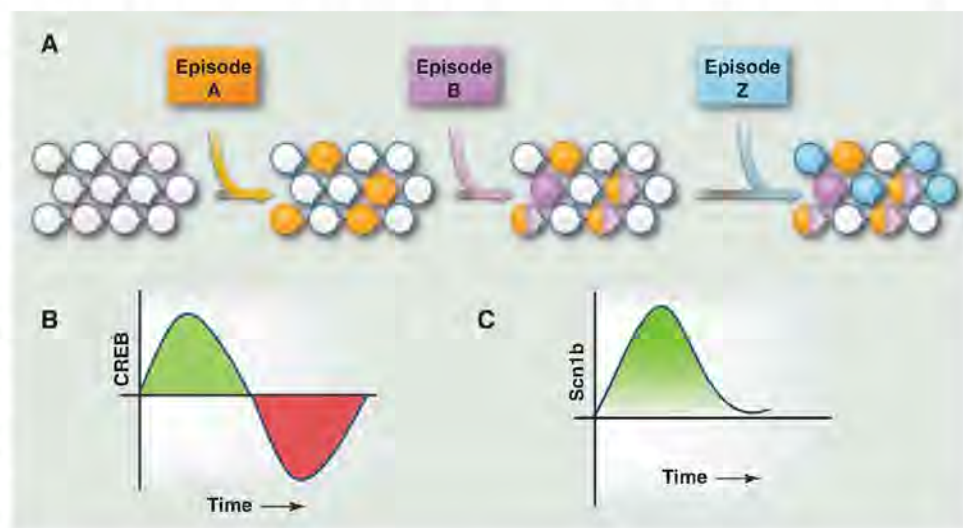


Fig. 1. Model of memory allocation in neuronal populations. (A) Neurons in a naïve neural circuit (open circles) are recruited into encoding episode A (orange). This increases their excitability so that shortly thereafter, they are very likely to also be involved in encoding episode B (purple). With time, the increase in excitability wanes, and consequently, episode Z (blue) is no longer stored in the same neurons. A consequence of this pattern of storage is that recall of episode A will also result in the recall of episode B (and vice versa), whereas recall of episode Z becomes unrelated to the other two episodes. (B) Training causes a temporary increase in the activity and levels of CREB. The CREB gene family of transcription factors includes both activators (they increase transcription) and repressors. After the initial increase in activators (green section of the curve), CREB repressors are expressed that decrease the overall levels of CREB activity, thus eventually bringing them below basal levels (red section of the curve). (C) Higher levels of CREB in a specific cell population result in increases in the levels of specific proteins (e.g., *Scn1b*) that, in turn, increase the excitability of neurons. Thus, two memories acquired while CREB levels are high would be stored in overlapping populations of neurons. Because the change in excitability would involve transcription and require the stability of transcribed molecules, such as channels, the time scale of this memory allocation mechanism would be on the order of hours (or perhaps even days), so that one memory could affect the allocation of the proceeding memories for many hours.

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in memory for tone fear conditioning (3). In this form of Pavlovian conditioning, animals learn to associate a tone with an aversive event, such as a mild foot shock. The amygdala is a subcortical brain structure with a well-known role in emotional memories such as fear memory (6, 7). Activity-regulated cytoskeletal (ARC)-associated protein RNA, a gene required for synaptic function and memory, was used to identify the neurons encoding a tone conditioning memory. ARC has been extensively used to determine which neuronal populations are activated by specific behavioral stimuli (8, 9). Immediately after a memory test, amygdala cells transfected with the viral CREB (identified through its fluorescent tag) were three times more likely to express ARC RNA (i.e., to be involved in memory) than were neighboring cells (3). Thus, CREB levels seem to bias which neurons encoded tone conditioning in the amygdala. It is conceivable that an interaction between the effects of the viral vectors used and the genes transfected could have affected the results of these early studies. To address this and other possible confounding interpretations of the results, cell lesion and inactivation strategies were also used to probe the role of CREB in memory allocation (4, 5).

A targeted cell lesion strategy was used to further explore the possibility that CREB biases the allocation of memory for tone conditioning in the amygdala (4). Han *et al.* took advantage of a transgenic mouse with a silenced diphtheria toxin receptor to specifically kill the cells with the virally delivered CREB in mice. A replication-defective herpes viral vector carried CREB and a recombinase that could activate the silenced diphtheria receptor gene by deleting a RNA translation stop sequence. CREB biased memory allocation because killing the cells transfected with the viral CREB disrupted memory for tone conditioning, whereas ablating the cells transfected with the control virus had no effect. A series of elegant control experiments showed that ablating the CREB cells did not prevent the animals from making new amygdala-dependent memories, nor did it affect memories acquired before viral infection. Instead, killing the cells with the viral CREB only affected the memory for tone conditioning acquired after viral transfection.

Targeted killing of nearly 20% of cells in a neural circuit may have had unintended effects that could confound the interpretation of behavioral experiments. However, this does not seem to have been the case, as similar results (5) to those just described were obtained with another approach (10) that allows for reversible neuronal inactivation. This strategy takes advantage of a *Drosophila* receptor (the allatostatin receptor) that can be functionally linked to potassium channels in mouse neurons. When activated by the allatostatin receptor, these channels silence neurons (i.e., keep them from firing action potentials). Because mice lack the ligand for the

allatostatin receptor, only neurons that have the exogenously expressed receptor can be inactivated by treatments with the allatostatin peptide. The findings (5) show that inactivating amygdala cells expressing a virally provided CREB results in a pronounced amnesia for tone conditioning. This amnesia could be readily reversed simply by retesting the mice 1 day later in the absence of allatostatin, demonstrating both the reversibility of the allatostatin effects and the link between activity in the CREB cells and recall. As before, if CREB was replaced with a control gene in the viral vectors, then inactivation of the transfected cells had no impact on memory. Further, the authors showed that CREB manipulations could also affect the allocation of memory for conditioned taste aversion (CTA) (5), another form of memory that engages the amygdala. Animals that experience intestinal malaise (i.e., nausea) after eating or drinking an unfamiliar food learn to avoid that food because they associate it with the subsequent malaise. Inactivating the neurons transfected with the CREB virus had a very specific impact on memory, because inactivation affected a CTA memory acquired after viral transfection, but did not disrupt in the same animals a tone conditioning memory acquired before viral transfection.

Each of the strategies used to study the role of CREB in memory allocation has its own limitations. For example, the expression of the allatostatin receptor could interfere with other receptors in amygdala neurons and would thus alter the behavioral results described above. Nevertheless, the convergence of findings with three different strategies (ARC cell tagging, diphtheria inactivation, and allatostatin inactivation) confirms that CREB has a role in the allocation of memory in the amygdala.

One of the by-products of the inactivation experiments described above (4, 5) is the finding that deleting or inactivating 15 to 20% of cells in an amygdala memory trace does not seem to affect that memory, whereas deleting 50 to 60% of those cells does cause substantial amnesia. This resiliency could be a mechanism for preserving memories in the face of neuronal death or other events that would otherwise erase memory traces.

The studies described above also suggest that there is a competition process that keeps the number of neurons encoding a given memory constant (3, 4, 11). Despite 60 to 70% of CREB-transfected cells being ARC positive and hence engaged in memory (versus 15 to 20% normally), the overall number of ARC positive cells in the amygdala did not increase. Therefore, it is possible that there are memory allocation mechanisms that control the size of memory traces so as to economize storage space. For example, changes in synaptic inhibition in response to changes in overall levels of circuit excitability could provide a dynamic way to control the overall number of neurons involved in a specific

memory, thus modulating the storage space allocated to any one memory.

Memory Allocation: Physiological Mechanisms

Electrophysiological studies (5) uncovered a possible mechanism for how CREB manipulations affect memory allocation. Consistent with previous studies (12), the findings showed that the cells transfected with the viral CREB were more excitable than neighboring cells or cells transfected with a virus carrying a control gene: The CREB cells fired more action potentials and were activated more easily (5) than neighbor or control cells. Perhaps because CREB cells are more excitable than these other cells, they are also more likely to respond to sensory inputs and therefore be activated during conditioning. Previous studies have suggested that learning triggers a temporary increase in neuronal excitability (13, 14). This excitability increase could define a window of time in which related memories are co-stored in overlapping populations of neurons.

The synaptic inputs carrying information about tone and shock are known to change the strength of synapses of neurons in the lateral amygdala (15). Electrophysiology studies showed that after tone conditioning, the potentiation of lateral amygdala inputs is considerably larger in the cells transfected with viral CREB than in their neighbors (5). A previous study (16) suggested that after conditioning, as many as 4% of all synapses can become potentiated in neurons recruited into amygdala memory traces. Together, these findings indicate that amygdala cells transfected with viral CREB are more excitable than their neighbors and express higher levels of synaptic potentiation after training, a result consistent with the hypothesis that these cells are preferentially chosen during tone conditioning. However, it is unlikely that CREB alone regulates which cells are involved in a given memory. Instead, it is plausible that the recent activation history of the cell (Fig. 1), as well as a myriad of molecular processes, including CREB targets, modulate memory allocation. As outlined in Fig. 1, CREB-dependent changes in excitability would involve a time scale of hours, so that one memory could affect the allocation of proceeding memories for hours afterwards. It is possible that other cellular allocation mechanisms would have different time scales. Next, we will review a series of findings that suggest that neurogenesis in the dentate gyrus may affect memory allocation with a time scale of days.

New Neurons and the Temporal Integration of Hippocampal Memories

The adult brain continues to make new neurons (neurogenesis), and this seems to be important for a number of processes, including learning and memory (17). Similar to the CREB-expressing amygdala neurons (5), newly born neurons in the

dentate gyrus of rodents show high levels of excitability (18, 19) and synaptic potentiation (20). Neurobehavioral (21) and computational (22) studies proposed that upon joining dentate gyrus circuits, these newly born neurons are more likely to be recruited into emerging memory traces, thus suggesting that neurogenesis has a role in memory allocation in the dentate gyrus.

A recent study (21) used 5-bromo-2'-deoxyuridine (BrdU), a permanent stain that intercalates dividing DNA, to label new neurons and genes known to be activated by training (*c-fos* and *ARC*) to determine whether these newly born neurons were engaged in spatial learning. The results (21) dem-

onstrated that 4- to 8-week-old neurons (BrdU+) are two to three times more likely than old neurons to be recruited into spatial memory (*c-fos*+ or *ARC*+). In contrast, 2-week-old neurons integrate into spatial memory circuits with lower efficiencies, and 1-week-old neurons did not integrate at all (21). These and other results showed that the timing of neuronal development relative to training is crucial, and they are consistent with previous studies showing that 4-week-old (but not 1-week-old) neurons have the required synaptic structures and physiology to support mature connections with established hippocampal circuits (23). Once mature, the newly born neurons are thought to receive connections from the entorhinal cortex and send connections to the

CA3 region. These results suggest that neurogenesis may affect memory allocation: Young dentate gyrus neurons are preferentially recruited after spatial learning. Recent computational modeling studies also suggested that neurogenesis has a role in memory allocation (22, 24). The authors designed a model network that incorporated key specific neuroanatomical features of the dentate gyrus, as well as immature neurons that matured progressively over time and became connected and fully functional. As in the dentate gyrus, new neurons in the model were temporarily endowed with the highest levels of excitability (18, 19) and synaptic

corporate a newer set of memories, each set with a distinct time stamp. Thus, new neurons with high excitability and plasticity could endow the dentate gyrus with pattern integration abilities. This idea for the temporal integration of memories does not necessarily contradict a role for the dentate gyrus as a pattern-separation device: Memories could be made distinct from other similar memories in the dentate gyrus by the million-plus mature neurons in this structure [whether they were born during development or in adult animals (26)] at the same time as distinct memories are given a specific time stamp (i.e., integrated) by newly connected young neurons (21). Altogether, the neuroanatomical and modeling studies described here suggest that neurogenesis has a role in memory allocation. Nevertheless, the findings could have alternative explanations. For example, the connectivity of new neurons, rather than dynamic memory cell-allocation mechanisms, could be responsible for their preferential incorporation into new memories. This connectivity, however, could also have a role of its own in memory allocation, with only a subpopulation of some of the synapses initially involved eventually being recruited into memory storage. Below, we will review recent results that suggest a synaptic allocation mechanism with a time scale of minutes.

Synaptic Selection and Memory Allocation

Although our descriptions of memory allocation thus far have focused on individual neurons, memories are thought to be stored in the synapses of those neurons (27, 28). Hence, single neurons are likely to be involved in multiple memories. Previous studies in the amygdala suggested that individual memories can recruit as much as 20% of all synapses of neurons engaged in that memory (16). Is the allotment of this synaptic space random, or are there mechanisms that determine synaptic selection during memory allocation? Previous synaptic-tagging studies showed that there are molecular physiological rules that determine which synapses are stably potentiated, suggesting that the commitment of specific synapses to memory is not random. Molecular tags may temporarily mark synapses activated during learning so that transcriptional products coming from the cell body stabilize synaptic changes specifically in those synapses. (29–31).

As predicted by clustered-plasticity models (31–33), long-term potentiation at a specific synapse in a CA1 pyramidal neuron increases the probability for potentiation at neighboring synapses. This affects synapses within 10 μ m of the potentiated synapse, and the effect may last at least 10 min (Fig. 2) (34). Potentiation of one synapse did not change the responses or structure of nearby synapses. Instead, it simply lowered the threshold for the induction of these changes in those synapses. In addition, potentiation of single synapses broadened the time window effective at

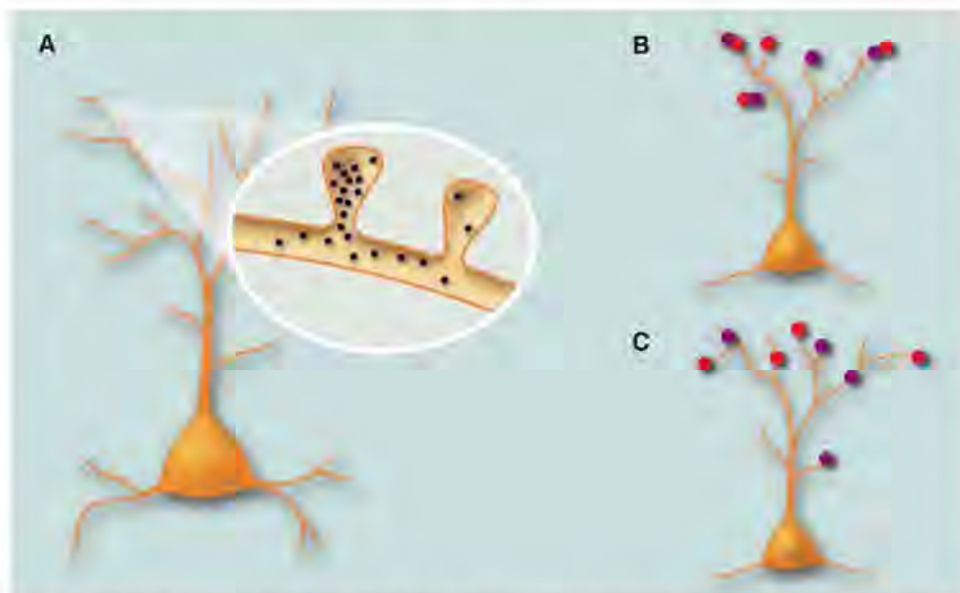


Fig. 2. Model of memory allocation within dendritic trees. (A) After learning and subsequent potentiation of synapses in encoding neurons, molecular components diffuse to nearby (10 μ m) unpotentiated spines for a limited time (10 min), thus resulting in a temporary increase in the probability that these spines will participate in subsequent learning (i.e., become potentiated themselves). (B) Two memories acquired within minutes of each other may be stored in similar populations of cells (Fig. 1) and in nearby synapses (red and purple circles), thus resulting in strong co-recall. (C) In contrast, two memories acquired within hours of each other may be stored in overlapping cellular populations, but perhaps not in nearby synapses, which would result in weaker co-recall. Lack of neuronal or dendritic colocalization would prevent automatic co-recall. Thus, unlike the cellular allocation mechanism discussed in Fig. 1, the time scale of synaptic allocation mechanisms would be on the order of minutes.

plasticity (20, 25). When the excitability and plasticity of a specific set of new neurons is high, they are very likely to participate in the encoding of incoming memories. Thus, a defined population of newly born neurons would encode and therefore integrate a set of memories occurring within a limited window of time. Later, activation of those new neurons could activate that specific set of memories. Once the excitability and plasticity of a specific group of newly born neurons wane, the window for memory integration closes and, consequently, a newer cluster of memories would be integrated into another group of maturing neurons. This process continues, with each subsequent set of newly born dentate neurons in-

tegrating a newer set of memories, each set with a distinct time stamp. Thus, new neurons with high excitability and plasticity could endow the dentate gyrus with pattern integration abilities. This idea for the temporal integration of memories does not necessarily contradict a role for the dentate gyrus as a pattern-separation device: Memories could be made distinct from other similar memories in the dentate gyrus by the million-plus mature neurons in this structure [whether they were born during development or in adult animals (26)] at the same time as distinct memories are given a specific time stamp (i.e., integrated) by newly connected young neurons (21). Altogether, the neuroanatomical and modeling studies described here suggest that neurogenesis has a role in memory allocation. Nevertheless, the findings could have alternative explanations. For example, the connectivity of new neurons, rather than dynamic memory cell-allocation mechanisms, could be responsible for their preferential incorporation into new memories. This connectivity, however, could also have a role of its own in memory allocation, with only a subpopulation of some of the synapses initially involved eventually being recruited into memory storage. Below, we will review recent results that suggest a synaptic allocation mechanism with a time scale of minutes.

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inducing potentiation in nearby synapses (34). These findings may have implications for memory allocation because they suggest that events closely associated in time (within 10 min of each other) are likely to be allocated (i.e., trigger synaptic modifications) to neighboring synapses in recruited CA1 neurons (Fig. 2). How could the potentiation of one synapse affect other nearby synapses?

Studies that fluorescently labeled and imaged specific biochemical changes in potentiated synapses demonstrated that some activated molecular components from one potentiated synapse can reach other nearby synapses (35), whereas others are spatially restricted to the activated synapses (36, 37). Because only some of the activated molecular components of potentiated synapses are shared, this could account for why potentiation is synapse-specific while affecting the probability of potentiation at nearby synapses. Branch-specific changes in excitability (38) could also affect synaptic selection mechanisms and therefore modulate memory allocation.

The results reviewed above are exciting, but it is important to note that the groundbreaking methods used to potentiate and image synapses could have affected the very biochemistry that they were intended to study. Furthermore, it remains to be shown whether similar mechanisms are at play during learning and memory. Nevertheless, these and other related results suggest that there are specific rules that regulate synaptic selection. Thus, besides mechanisms that determine which cells are engaged in a given memory, working with time scales of hours and days (Fig. 1), there may be also mechanisms, functioning with time scales of minutes, that determine which synapses are recruited. What would be the implications of having allocation mechanisms with different time scales? One possibility is that cell and synaptic allocation mechanisms could set up gradients of memory relatedness: Memories with both overlapping cellular and synaptic domains would be expected to be more interconnected (more often co-retrieved) than memories that only share similar cell populations (Fig. 2B). The very different time scales of these processes would limit memory integration at the synaptic and cellular levels. Accordingly, synaptic integration would require closer juxtaposition of events (seconds and minutes) than cellular allocation (hours and days).

The Allocation of Episodic Memories

Episodic memories usually involve extended temporal interconnections between complex stimuli. Memory allocation mechanisms could potentially be used to set up partially overlapping populations of neurons that could account for these extended temporal interconnections. However, previous studies showed that very specific features of complex stimuli only briefly activate neurons in the human hippocampus and asso-

ciated structures [such as the medial temporal lobe (MTL)]. Activation normally lasts 300 to 600 ms and rarely continues beyond 1 to 2 s after stimulus onset. However, episodic memories can be much longer, and it is not known how they are encoded in MTL. Are different components of an episodic memory allocated to overlapping populations of neurons, or are they encoded in separate MTL neural circuits? Consistent with the former possibility, prolonged activations of MTL neurons in response to single episodes have now been reported (39). The participants in these experiments were epileptic patients with implanted electrodes in the MTL to help locate the source of seizures. To trigger more extended episodic memories, participants were initially exposed to 5- to 10-s clips of movies while implanted electrodes recorded responses from the MTL.

Nearly 10% of the 847 units recorded showed prolonged responses throughout the entire replay of the movie clip (i.e., during the whole episode). Each episode included numerous related but changing features that were nevertheless continuously recognized by single MTL neurons. Although there are many potential explanations for this prolonged firing, one possibility is that different episodic components present in different parts of the clip were allocated to the same neurons. Another possibility is that firing of the units recorded was not specific, which could account for continued firing during the entire clip; however, this was not the case. For example, one of the units in the entorhinal cortex fired specifically in response to a clip (among 48 clips) of an episode of the television series *The Simpsons*. Could the rules of cellular and synaptic memory allocation emerging from studies in rodents also apply to human memory? Could CREB-dependent mechanisms, neurogenesis, and synaptic selection have a role in human episodic memory formation? Studies like the one mentioned above could start to address these important questions.

Conclusions

Newly developed strategies for imaging, activating, and inactivating specific neurons in a given brain region are changing the way neuroscientists tackle traditional problems, and more importantly, they are opening up new areas of investigation, such as memory allocation. The results reviewed here represent the first steps toward uncovering the molecular and cellular strategies used by circuits to allocate information during encoding. They suggest that mechanisms working at different time scales (including CREB signaling, neurogenesis, and synaptic selection) modulate the allocation of memory to specific cells and synapses within a neural network. Memory allocation will probably involve a plethora of synaptic, cellular, and systems mechanisms regulated by a myriad of molecular processes, including CREB. Beyond CREB, it

is possible that other molecular components involved in the acquisition and consolidation of memory may also regulate which cells are allocated to a given memory, as well as which synapses in those cells are eventually recruited into encoding that information.

The results reviewed here suggest that there are competitive mechanisms that affect memory allocation. For example, new dentate gyrus neurons, amygdala cells with higher excitability, and synapses near previously potentiated synapses seem to have the competitive edge over other cells and synapses and thus affect memory allocation with time scales of weeks, hours, and minutes. Are all memory allocation mechanisms competitive, or are there mechanisms of memory allocation that do not involve competition? Even though it is difficult to resolve this question at the current time, it is important to note that most mechanisms of memory allocation in computers do not involve competition.

Studies of memory allocation may also have an impact on the study of memory deficits. For example, a number of studies have shown that aging decreases both the excitability of neurons in the hippocampal formation and the rates of neurogenesis. Consequently, aging very likely disrupts hippocampal memory allocation, which could possibly contribute to age-related cognitive decline. Disruptions of inhibition and modulatory neurotransmission in psychiatric conditions are also likely to affect memory allocation and, therefore, to be partially responsible for cognitive impairments associated with these conditions. Inappropriate associations between unrelated events could conceivably be a reflection of abnormal memory allocation in psychiatric conditions such as schizophrenia.

Although memory allocation has only been recently studied in neuroscience, there is a long history of studies of memory allocation in computer science. Insights into the strategies that allocate information in neuronal networks may prove useful for the treatment of memory disorders and suggest innovative design principles for effective allocation of memory in artificial networks.

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REVIEW

The Optogenetic Catechism

Gero Miesenböck

An emerging set of methods enables an experimental dialogue with biological systems composed of many interacting cell types—in particular, with neural circuits in the brain. These methods are sometimes called “optogenetic” because they use light-responsive proteins (“opto-”) encoded in DNA (“-genetic”). Optogenetic devices can be introduced into tissues or whole organisms by genetic manipulation and be expressed in anatomically or functionally defined groups of cells. Two kinds of devices perform complementary functions: Light-driven actuators control electrochemical signals, while light-emitting sensors report them. Actuators pose questions by delivering targeted perturbations; sensors (and other measurements) signal answers. These catechisms are beginning to yield previously unattainable insight into the organization of neural circuits, the regulation of their collective dynamics, and the causal relationships between cellular activity patterns and behavior.

What Is Optogenetics?

The 2019 revision of the Oxford English Dictionary, which may be the first to recognize the new word, will define optogenetics as “the branch of biotechnology which combines genetic engineering with optics to observe and control the function of genetically targeted groups of cells with light, often in the intact animal.” Although the foundations of the field were laid in the late nineties and early naughts (1–12), the term appeared in the literature only in 2006 (13). Purists have remarked that “optogenetics” is a misnomer: similar coinages, such as optoacoustics or optoelectronics, refer, respectively, to interactions of light with sound and electrons. Optogenetics, by contrast, has nothing to do with interactions between light and genes; what matters is the effects of light on the protein products of genes.

What Kinds of Light-Sensitive Proteins Are Used in Optogenetics?

There are two classes of optogenetic devices (Fig. 1): sensors and actuators (1). Sensors trans-

late cell physiological signals into optical signals; they make cellular function visible. Actuators transduce optical signals into physiological signals; they make cellular function controllable. The pleasing symmetry between sensing and actuation is practically important because sensors and actuators together make up a complete experimental package: Actuators deliver controlled perturbations, and sensors report system responses back.

Experimentation on Which Systems?

Optogenetics was developed to study information processing in the brain (1, 2, 13), but it is certain to find many applications outside neuroscience. One vast, still entirely unexplored territory is information processing in the immune system.

What Is Special About Constructing Light-Emitting Sensors and Light-Driven Actuators from Proteins?

Proteins can be encoded in DNA. DNA molecules are stable, portable pieces of code that can be packaged into many different kinds of delivery vehicles and integrated into the genome of nearly any organism. Once a piece of DNA has

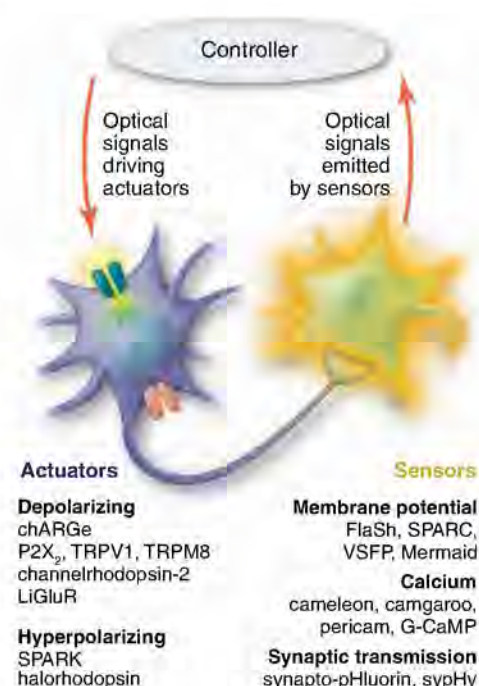


Fig. 1. Sensors and actuators. Light-driven actuator proteins are used to control genetically targeted cells in a circuit. The actuators transduce optical commands into de- or hyperpolarizing currents. Light-emitting sensor proteins report changes in membrane potential, intracellular calcium concentration, or synaptic transmission.

been introduced into a cell, endogenous machinery is directed to produce the required protein. This solves the problem of delivering experimental agents deep into the tissues of intact organisms: After genetic modification, the organism itself generates the tools necessary for investigating its function; biology is revealed through biology.

However, not all optogenetic devices are wholly encodable. Some sensors (3, 14) and actuators (8–10, 15, 16) depend, in addition to a genetically encoded component, on small molecules that must be fed or injected. Others (11, 12, 17–19) require protein expression levels so high that they cannot routinely be achieved by

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modifications of the host genome, forcing once again a resort to invasive procedures with variable penetrance: the injection of plasmids (20–22) or viruses (23–25).

Although localized agent delivery can enhance the region selectivity of expression in some circumstances (20, 22, 26), these characteristics generally undermine the elegant economy of a stably heritable, fully biosynthetic system. The need to manipulate each experimental subject individually reduces throughput, which makes optogenetic screens, if not impossible, certainly laborious. It also introduces a new source of variability, which must be controlled.

However, the delivery problem is not the only, and not the most important, impediment that a optogenetics was developed to overcome. By encoding sensors and actuators in DNA, their distribution can be restricted to particular cell types, tapping the same mechanisms that localize the expression of endogenous genes. Gene expression patterns thus provide a natural framework for exploring biological function.

Do Gene Expression Patterns Meaningfully Reflect the Functional Organization of Cells into Tissues?

Yes, but single genes are often either too loquacious or too reserved in their expression. Most isolated enhancers yield expression patterns that match behaviorally or physiologically relevant subsets of neurons only approximately; coverage is often insufficiently inclusive, inadequately exclusive, or both (Fig. 2). This is not surprising: Cell identities are thought to be specified combinatorially, by using a regulatory syntax understood only incompletely. We know, for instance, a genetic label for “dopaminergic” in invertebrates; it is “a cell in which the tyrosine hydroxylase gene is turned on.” Exploiting this knowledge (and randomly some of the influence exerted on transgene expression by genomic context) made it possible to control remotely different subsets of dopaminergic neurons in the behaving fly (10, 27). The origin of reinforcement signals driving adaptive behavior could thereby be mapped to a cluster of 12 dopaminergic cells—one of two existing examples of small-scale optogenetic screens (27, 28). But attempts to target these cells selectively among the 200 or so other dopaminergic neurons in the fly brain failed because of an inability to translate attributes like “source of reinforcement signals,” “located in the PPL1 cluster of dopaminergic neurons,” or “projecting to the mushroom body” into the language of gene expression.

To take full advantage of optogenetic tools, it is essential that they can be targeted selectively and comprehensively to the cells of interest. The capacity to do so remains limited, as discriminating audiences appreciate all too well. Improving access to identified cells is as much a matter of expanding the genetic lexicon—that is, of associating as many molecular descriptors as possible

with each identified cell—as it is of designing boolean operators (1, 29) that can decode sets of such descriptors as unique cellular addresses.

Which Cell Physiological Signals Are Sensed?

An engineer thinking about sensor placement in a living cell might despair after a casual look at a chart of signaling pathways. Closer scrutiny, however, will reveal structural simplicity within the apparent complexity. Many signaling molecules are wired together in modules with identifiable functions, and many of these modules operate in distinct temporal frequency bands. The low-frequency band, for example, contains slow developmental, growth control, and differentiation programs that regulate, by and large, gene expression. Distinct from these are the high-frequency signals that flow through neural, immunological, and endocrine circuits and convey information about rapidly varying internal and external states.

exist (4–6) (Fig. 1). In addition to these generalists, there is also an ever-expanding range of specialist probes for analytes such as adenosine 3',5'-monophosphate (cAMP) and for processes such as cell cycle progression (30).

How Do Sensors Work?

Virtually all optogenetic sensors are derivatives of the green fluorescent protein (GFP), engineered to modulate their light output in response to a physiological variable. One of the two common mechanisms of modulation is chromophore protonation-deprotonation cycles, which are typically driven by conformational changes that allow or restrict solvent access to the chromophore. The second mechanism is variations in the strength of electrodynamic interactions between nearby chromophores, caused by changes in proximity and/or dipole orientation. The calcium sensor G-CaMP illustrates the first mech-

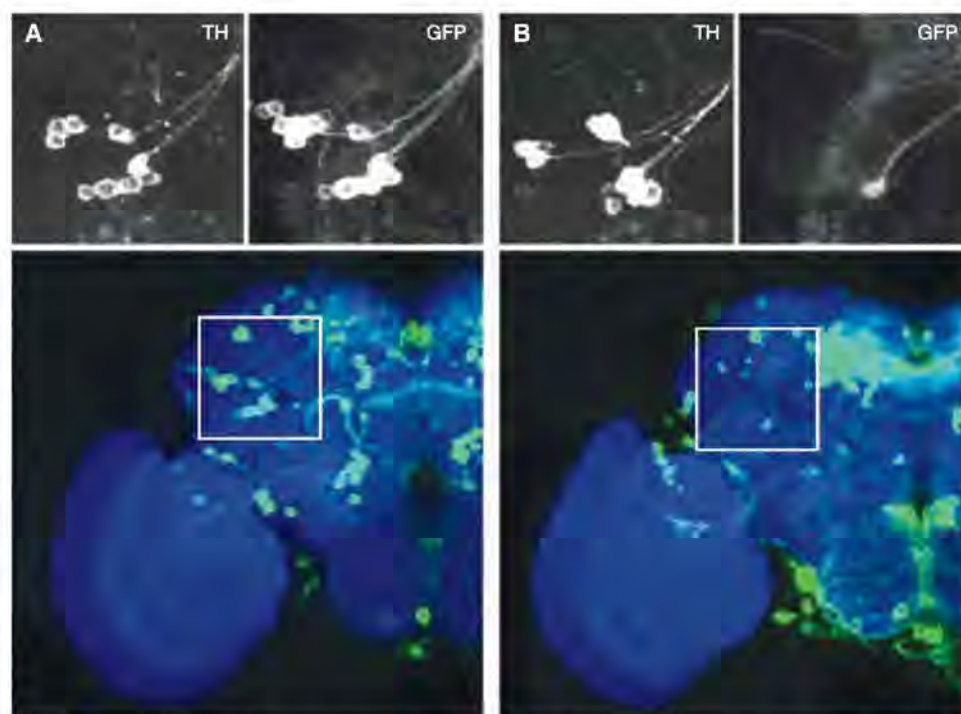


Fig. 2. Genetic access to neurons in the fly brain. Two different enhancer elements are used to drive the expression of GFP. The two enhancers each label neurons in the same dopaminergic cluster, which is visible in the left top images after staining with an antibody against tyrosine hydroxylase (TH). The enhancer in (A) labels the cluster exhaustively, whereas the enhancer in (B) targets a single identified neuron in it. Views of the whole brain (bottom images) reveal that neither enhancer expression pattern exclusively captures the dopaminergic neurons of interest.

Although there is much diversity in the molecular carriers of these messages along a communication chain, many of them are eventually translated into a cellular Esperanto of membrane potential changes, calcium admission from extra- and intracellular reservoirs, and secretory events triggered by calcium. The most versatile sensors are those that detect and report these changes.

Protein-based optical sensors for the three key variables of voltage, calcium, and secretion now

anism, the calcium sensor cameleon the second (1, 30, 31).

How Well Do Sensors Perform?

A biophysicist's answer will be framed in terms of the sensor's response kinetics, dynamic range, absolute brightness, photostability, specificity, the potential for perturbation of cellular function, and so forth. Because many of these parameters are in conflict with each other, sensor optimization tends

to force substantial trade-offs; these and the signal detection theory behind them are discussed elsewhere (1, 29–32). As a simple illustration, consider the problem of optically detecting a neuronal action potential (5, 32). Naïvely, one might suppose the best approach to consist of packing the neuron's cell membrane densely with the fastest possible voltage sensor. However, resolving the light emissions of individual neurons in thick, scattering tissue generally requires some form of scanning microscopy, which samples each spatial location only intermittently. This has two important consequences.

First, to avoid detection failures while looking elsewhere, each location must be revisited at intervals shorter than the typical duration of an optical event. To detect an action potential of 1-ms duration by using a membrane potential sensor with a fast impulse response, the sampling rate must exceed 1 kHz. This limits the number of cells that can be surveyed.

Second, short events have few photons associated with them, making detection statistics unfavorable. The problem could, in principle, be ameliorated by expressing more sensor molecules, but this increases the risk of sometimes violent interference with the variable of interest. Such an observer effect plagues the expression of genetically encoded voltage sensors: When these probes are present at the levels required for action potential detection, they suppress synaptically evoked electrical activity (14). The culprit is the capacitive load added by the mobile probe charges sensing the cellular membrane potential (14, 32).

Can Optogenetic Sensors Detect Naturalistic Activity Patterns in Brain Circuits?

No. All optical sensors are fundamentally limited by their photon statistics. The circle of collecting sufficient photons from many optically resolved cells at high sampling rates, so that activity can be detected reliably, has not been squared, not with synthetic and not with optogenetic probes.

Existing encodable voltage probes (5, 14) interfere with electrical excitability (14, 32). Calcium (4) and exocytosis sensors (6) are better tolerated than voltage probes and able to resolve single isolated action potentials and synaptic impulses under favorable conditions. However, they still lack the temporal resolution to distinguish such events in naturalistic settings (1, 29, 30).

Even though functional optical imaging is so far unable to keep pace with dynamics on electrophysiological time scales, it offers insights into the organization of cells and systems that would be difficult to obtain by other means. Maps of orientation or direction selectivity in visual cortex could, in principle, be pieced together from hundreds of single-unit recordings, but only functional images reveal at once, in one individual, the geometry and self-similarity of these maps across macroscopic and microscopic scales

(33). The genetic resolution of encodable sensors makes them ideal for eavesdropping at the mezzanine level: They can discriminate how macroscopic order emerges from layering and interactions of distinct groups of microscopic elements (34–36).

Are Optogenetic Actuators Able to Control the Key Cellular Information Carriers: Voltage, Calcium, and Secretion of Signaling Molecules?

In broad outline, yes. Because the majority of actuators (Fig. 1) are ion channels (8–10, 15), ion pumps (17–19), or proteins that regulate ion channels (7), they are able to influence transmembrane voltage. Many of these channels and pumps—TRPV1 (8), TRPM8 (8), P2X₂ (8), channelrhodopsin-2 (ChR2) (11, 12, 17), and the light-gated glutamate receptor (LiGluR) (15)—conduct calcium ions (37), so they can also control calcium-dependent processes, including regulated secretion. Because calcium influx and exocytosis are tied to voltage changes, it is not usually feasible to regulate these processes independently: to elevate intracellular calcium levels without altering membrane potential or to trigger secretion directly.

As in the sensors' case, there are also specialized actuators, such as light-activated adenylyl cyclases, transcription factors, kinases, and heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors. Biodiversity prospecting and bioengineering will undoubtedly extend this range further.

How Is Light Sensitivity Built into Actuators?

In actuators regulating membrane potential, photons exert force by driving chemical changes in either retinal or exogenous light-sensitive small molecules (1, 37). Retinal, an endogenous vitamin A derivative, functions as the chromophore in two large families of optically responsive proteins, known as opsins in free form and as rhodopsins when in complex with retinal. The visual rhodopsins of vertebrate and invertebrate eyes are G protein-coupled receptors; when illuminated, they engage intracellular signaling cascades that control the opening or closing of ion channels in the plasma membrane or in intracellular compartments (7, 12).

Microbial rhodopsins, in contrast, are light-driven proton or chloride pumps, which couple the photocycle of retinal to the translocation of ions (37). When coupling is leaky, as in ChR2 (38), the pump cycle includes passively conducting intermediates: The pump functions (intermittently) as a nonselective channel (17).

The alternative to retinal as a light-operated lever is irreversible photolysis (so-called uncaging) or reversible photoisomerization of synthetic photochemicals. Specificity of action is ensured in two ways. One is orthogonality: The photolytically liberated compound lacks activity against endogenous targets and thus requires the expression of an exogenous receptor (8, 10). The other mechanism is recruitment: The activity of a

broadly active photoswitchable ligand is localized (and potentiated) by docking the molecule to an adhesive patch on the actuator protein (9, 15, 26).

How Well Do Actuators Work?

A biophysicist will look to several performance indices: the actuator's single-channel conductance, its response kinetics, the open probability in the dark, cofactor requirements, the capacity to regenerate the optically responsive state, the potential for controlling on and off transitions independently, and the precision with which the optical signal driving the actuator can be localized in scattering tissue. As in the sensors' case, there are substantial trade-offs to consider and a growing literature to help weigh them (1, 26, 29, 37).

Perhaps the most important balance to strike is between actuator force and speed. Unsurprisingly, the tighter the mechanistic link between photochemical process and ion flux, the faster the actuator's kinetics. Actuators that are gated by diffusible intermediates, whether products of signal transduction pathways (7, 12) or photolysis (8, 10), respond with temporal precision ranging from ~10 ms to seconds. Actuators with built-in chromophores, such as ChR2 (11, 12, 17), LiGluR (15, 16), and halorhodopsin (18, 19), can be controlled with higher millisecond or submillisecond accuracy. Unfortunately, however, the fastest actuators are also the weakest: The unitary conductance of ChR2 is only 40 to 50 fS (17, 38) and that of LiGluR, 250 fS (16, 26); TRPV1 and P2X₂, by comparison, have conductances of 30 to 35 pS at resting potential (8, 37). This nearly 1000-fold difference in the force exerted by a single actuator molecule explains the ineffectiveness of microbial opsins in some settings (for example, the intact adult fly central nervous system) and the requirement for massive overexpression in many others.

What Have Actuators Been Used for?

The earliest applications of encodable actuators were naturally proof-of-principle experiments: demonstrations that remote activation of neurons known to control particular functions produced the predicted outcomes (10, 18, 23). Many recent studies have continued in this vein, voyaged into the known, and discovered the expected. Where truly novel insights have emerged, they tend to fall into three areas: tracing of functional connections (20, 22), analysis of mechanisms by which neural circuits (self-) regulate their activity (24, 25, 28, 39), and searches for the neural underpinnings of cognition and behavior (21, 24, 27, 28, 39).

An example from the first category is the demonstration that inputs from ascending, local, and descending axons are segregated onto discrete dendritic domains of neocortical pyramidal cells (22). Transmitting information through synaptic clusters rather than distributed connections may result in more efficient coupling between pools of synaptic partners. One (perhaps rather

too high) estimate suggests that at most 300 action potentials in layer 2/3 pyramidal cortical neurons represent a basic parcel of propagating activity (21).

An advance in the second category, insight into mechanisms of neural control, is the discovery that animals harbor functional circuits for behaviors they never express. The smoking gun is a cryptic capacity of female fruit flies for displaying male-specific courtship (39). This hidden talent was uncovered after optical activation of neurons expressing a principal sex determination factor. Latent bisexuality may be a vestige of shared brain development in males and females, on which sex-specific action selection mechanisms are later superimposed.

In the third category, even the neuronal substrates of some higher-order cognitive functions, such as the construction of valuations (27) or the gating of sensory responses (24), have been delineated. They include not only narrowly circumscribed spatial (27) but also distinct temporal domains (24) of activity.

Is It Important for Artificial Actuation to Mimic Naturalistic Activity?

It is unlikely that it can, and it is not clear that it needs to or should. The reasons are manifold.

In practical terms, we do not know what a naturalistic neuronal activity pattern looks like because no one has been able to record one. We have many examples of isolated spike trains from different types of neurons and in different conditions. But what is generally lacking (with a few notable exceptions) is knowledge of the temporal relationships between the spike patterns of many cells during simultaneous activity. Probing the importance of these phase relationships in information processing is a key future application of optogenetic technology, one whose outlines are just beginning to appear (24, 25). But these experiments require bold systematic variation, not slavish imitation, of naturalistic activity.

Even if we knew the timing of every spike in every cell of a circuit, playing back such an activity pattern to a volume of tissue would be no simple technical feat. Setting aside questions about the efficiency and precise spatial addressability of optogenetic actuators, patterned illumination of hundreds to thousands of locations simultaneously but independently poses enormous challenges. These challenges are magnified because positive actuation alone—translating the desired spike pattern into a pattern of light pulses and delivering these pulses—will generate uncontrolled runaway behavior. To ensure stability and constrain the system to a prescribed trajectory, negative control signals will have to be applied in addition to positive actuation, just as de- and hyperpolarizing currents are both necessary for keeping a neuron under voltage clamp. Akin to a voltage-clamp amplifier, these control signals can only be determined if the system's behavior is sensed in real

time and fed back to the controller. The unsolved problem of densely sampling neuronal spike patterns at high temporal resolution again rears its head.

Empirically, even rather crude and highly artificial optogenetic actuation has proved remarkably effective. In many instances, firing a sizeable number of neurons at once or forcing a population of cells periodically produces, instead of chaos, coordinated behavior: flies fly (10) and court (39); fish swim (28); memories can be written (27); mice are nudged from sleep to wakefulness (23); decisions can be biased (21); cortical neurons synchronize (24, 25). This suggests that neural systems do not easily adopt any of the astronomical number of dynamical states that are theoretically open to them; rather, they appear to switch between a more limited repertoire of preferred activity patterns. After a perturbation, be it sensory input, motor command, update of the contents of working memory, or optogenetic intervention, the circuit settles into one of these so-called attractor states. Attractors can be thought of as minima in the free energy landscape characterizing the circuit's dynamics, with a surrounding basin of initial states that feed into them. The hallmark of attractor dynamics is that, irrespective of where exactly in the basin the circuit lands after a perturbation, its trajectory will evolve toward a common dynamical state. The unreasonable effectiveness of optogenetics in eliciting physiological responses hints that attractor dynamics is a common feature of brain circuits. Of course, testing this interpretation rigorously will require that the dynamic endpoints reached from many different initial states are mapped systematically.

This leads to the statistical argument that complex systems are best probed, not by replicating any one particular activity pattern, but by applying whole families of perturbations which may be unapologetically artificial. The core of this argument was articulated by R. A. Fisher (40): "No aphorism is more frequently repeated [...] than that we must ask Nature few questions, or, ideally, one question, at a time. The writer is convinced that this view is wholly mistaken. Nature, he suggests, will best respond to a logical and carefully thought out questionnaire [...]." Statisticians recognize Fisher's questionnaires, also known as randomized multifactorial perturbations, as the most powerful experimental design for uncovering causal relationships in networks of interacting components, and biologists are taking note. Geneticists, for example, increasingly seize the advantages of analyzing the simultaneous phenotypic effects of thousands to millions of allelic differences instead of looking, one at a time, at a collection of single-gene mutants. Neuroscientists have long embraced randomized multifactorial perturbations under the rubric of white noise analysis and related methods of systems identification in sensory physiology (41). The advent

of optogenetic control is an incentive to revive this tradition but also to step beyond single neurons and sensory systems and insert synthetic signals directly into defined neural elements deep in the brain. The development of stimulation protocols and technology that address many such elements with intelligently constructed test signals, such as spatiotemporal sequences, can provide a global characterization of responses over many possible inputs and thus expose a circuit's dynamic modes and their functional importance.

Why Not Just Watch?

Mechanistic understanding requires intervention. This is as true for a geneticist wishing to link a phenotype to a gene as it is for a biochemist seeking to purify an enzyme, and it is of course also true for a neuroscientist attempting to isolate the neural signals that drive behavior. The information-carrying features of these signals rarely pop out from recorded neuronal chatter. Rather, they are found by systematic deletion, variation, and reconstitution of activity. Even if observation suggests that information is represented in a particular aspect of a neuronal activity pattern, the leap from correlation to causality still demands experimental intervention (10).

It is difficult to imagine how the genetic code could have been broken by observation alone. The decisive advance occurred when the genetic codebreakers instructed ribosomes with simple synthetic messages (42). Optogenetic instruction of the nervous system can play a similar role in the quest to decipher neural codes.

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REVIEW

Modalities, Modes, and Models in Functional Neuroimaging

Karl J. Friston

In this, the 21st century, human-brain mapping celebrates 21 years of cognitive activation studies. This review looks at imaging neuroscience and key ideas it has pursued; some ideas portend exciting developments, and others have failed gloriously. In terms of achievements, there is much to celebrate, in the sense that it is difficult to imagine modern neuroscience without brain imaging. I will look at recent advances from the perspectives of functional segregation and integration in the brain, paying special attention to approaches that deal with the distributed and integrated nature of neuronal processing and the questions they address.

Neuroimaging is now the predominant technique in behavioral and cognitive neuroscience. The volume of papers and number of fields it pervades are unrivaled (Fig. 1). Despite this, it is curiously difficult to summarize its achievements. The simplest summary falls back on the two guiding principles that shaped brain mapping at its inception; namely, functional segregation and integration. Neuroimaging has established functional segregation (the segregated or modular deployment of functional specialization within brain regions) as a fundament of brain organization. Furthermore, we can now characterize the integration of different brain areas in terms of functional and effective connectivity (Fig. 2). But beyond this, what have we learned? If you ask any imaging neuroscientist, they will recount exciting developments in their own field, ranging from the detailed functional architecture of retinotopically mapped visual cortex to the role of the ventral striatum in emotional learning. However, the question is more difficult to answer in terms of generic principles that underlie the brain's function and its relationship to anatomy. To see how people have tried to access these broader principles, I will look at recent trends in func-

tional magnetic resonance imaging (fMRI), with a special focus on the questions that have been addressed. This focus is particularly important for functional neuroimaging, whose contributions will be measured by the depth of the questions asked, not the elegance of the method or, perhaps, the answers.

I first consider four themes that have caught people's imaginations recently and examine their underlying motivations, noting that there are many other exciting developments I could have addressed [such as genetics in neuroimaging, psychopharmacological fMRI, invasive and non-invasive electrophysiology, retinotopic mapping, computational anatomy, tractography with diffusion weighted imaging, lesion-deficit mapping, magnetic resonance spectroscopy, optical imaging, and technical advances such as polarization transfer (1)]. I then consider a couple of failures and conclude with a discussion of the implications for future directions; this discussion is illustrated with a few questions or model-led examples.

Multimodal Fusion

For years, we have heard about the promise of multimodal fusion, in which the spatial precision of fMRI will be complemented with the temporal precision of electroencephalography (EEG) to provide unprecedented spatiotemporal accuracy. However, this has not happened, despite the fact that we have the technology to acquire both mo-

dalities simultaneously (2) and have sophisticated biophysical models mapping from neuronal activity to both hemodynamic and electromagnetic measurements (3). So why is multimodal imaging not commonplace? Perhaps because there are many questions about functional anatomy that do not need bilateral spatial and temporal precision. Most questions about structure-function mapping and neuronal processing come in two flavors: where is it? or when is it? Functional magnetic resonance imaging is quite sufficient for questions of where and electromagnetic measurements [EEG and magnetoencephalography (MEG)] are the modalities of choice for questions of when; however, there are also questions about how imaging signals are generated that rest on fusion.

Multimodal fusion refers to the use of a common forward model of neuronal activity that explains different sorts of data. Several years ago, this was thought to be the best way to integrate fMRI and EEG because model parameters that are under-constrained by one modality might be informed by the other. In retrospect, this may have been a little misguided because the added value afforded by fusion requires unknown quantities generating data to express themselves in both modalities. Ironically, it may be that the complementary aspects of fMRI and EEG subvert the benefits of fusion. This may explain the success of simpler approaches to multimodal integration, in which the results from one modality constrain models of the other. These approaches use fMRI to provide precise spatial constraints (priors) on the source reconstruction of electromagnetic signals (4). Conversely, the temporal precision of EEG has been exploited in epilepsy research, in which explanatory variables based upon EEG features provide temporal constraints (in the form of explanatory variables or regressors) to model fMRI data (5).

So what questions call for fusion? A nice example is fusion of EEG and MEG data to measure evoked or induced responses, in which each modality alone is blind to certain sources (6). However, multimodal fusion really comes into its own when trying to understand the neurophysiology of brain-imaging signals and how they reflect underlying computations: Questions about func-

tional segregation are constrained by the resolution of fMRI. For example, a voxel (volume element of several mm^3) contains on average 5.5 million neurons, 10^{10} synapses, 22 km of dendrites, and 220 km of axons. Clearly, this precludes questions about functional specialization within the dendritic tree or indeed a cortical macrocolumn and calls for a broader notion of multimodal fusion, in which noninvasive imaging and invasive microscopic techniques are used to understand the principles of cortical computation that transcend spatial scales. I will return to this later but first consider techniques that try to find evidence for functional segregation at the voxel level.

Multivariate Pattern Classification, Decoding, and Mind-Reading

There has been immense interest recently in the use of multivariate pattern classification to infer the intentions or percepts of subjects by using fMRI measurements (mind-reading or decoding) (7–9). Conventional brain mapping tries to establish statistical dependencies between experimental manipulations and measured brain responses. This is related to a similar mapping in computational neuroscience, which refers to how neurons encode features in the outside world. The reverse mapping from measured physiological signals to the features encoded is called decoding. Decoding, reverse inference, or mind-reading uses multivariate analyses of fMRI data to classify the perceptual or cognitive state of a subject. Crucially, they harness patchy functional segregation (such as orientation selectivity in the visual cortex) at the voxel and subvoxel scale to search for patterns over voxels that best discriminate between experimental conditions.

Despite the allure of mind reading, one has to be wary of admiring the “emperor’s new clothes.” This is because multivariate pattern classification conflates multivariate with classification. Put briefly, their enhanced sensitivity and finessed characterizations of distributed responses rest on the use of multivariate models, not classification or reverse inference. Demonstrating a significant mapping between mental states and brain signals does not depend on the direction of the mapping (as with a significant correlation). In other words, showing that one can decode activity in the visual cortex to classify (above chance) a subject’s percept is exactly the same as demonstrating significant visual cortex responses to perceptual changes. In this sense, all demonstrations of functionally specialized responses represent an implicit mind-reading. So what are the new questions behind decoding studies? If one looks closely, the questions are the same as in conventional encoding analyses: Basically, are there regionally specific correlates of some cognitive, perceptual, or sensorimotor state? However, when addressed with multivariate analyses the question pertains to distributed neuronal activity. This is because

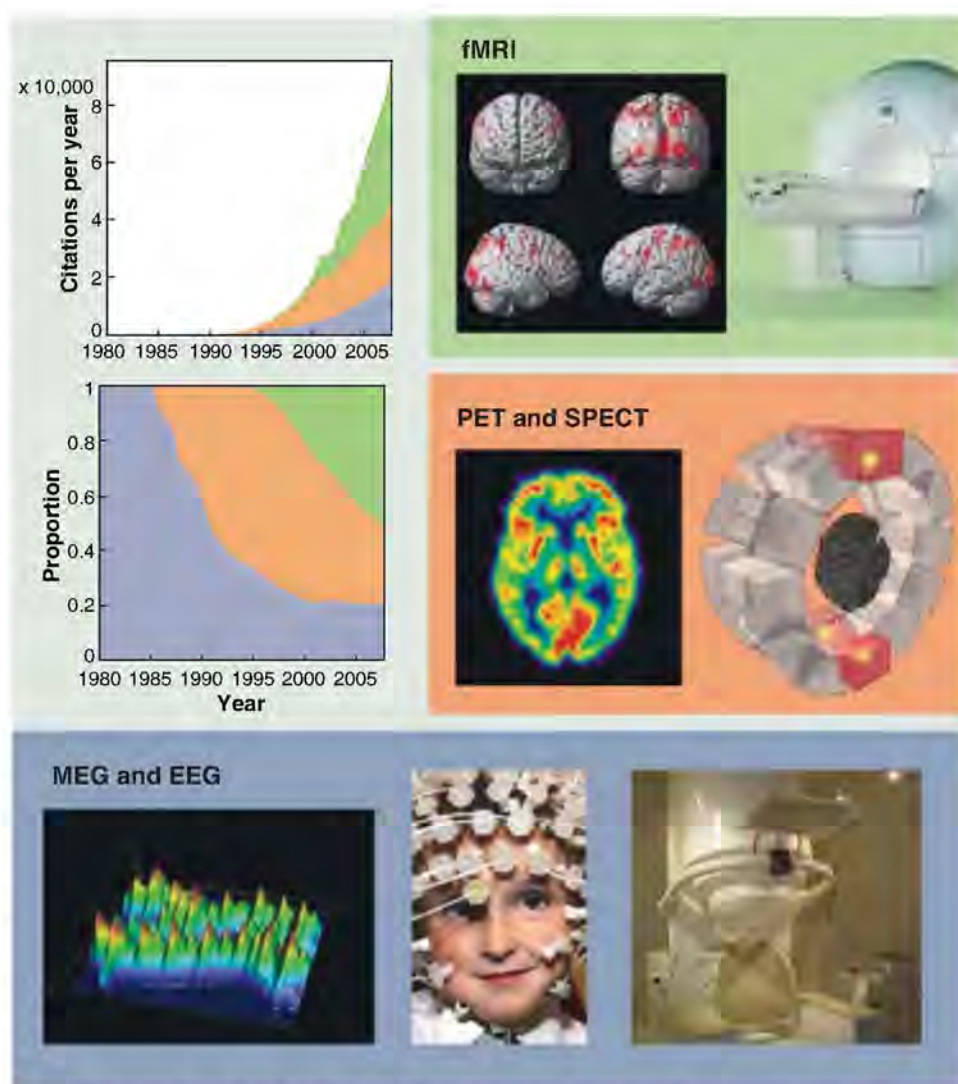


Fig. 1. Citation rates for the different modalities of functional neuroimaging. PET, positron emission tomography; SPECT, single-photon emission computed tomography. The citation rates [in 10,000 citations per year (**top left**) and proportion by modality (**middle left**)] are shown for the past 30 years. These data came from the ISI Web of Knowledge by searching for EEG OR MEG, PET OR SPECT, fMRI AND Brain with Topic = Neurosciences.

one is no longer testing for a dependency between an experimental variable and activity in one voxel but distributed responses over many voxels (Fig. 3). These distributed responses may have a fine-scale structure and be highly subject-specific, as opposed to the “blobs” identified in conventional mass-univariate analyses. This speaks to exciting developments, in which *t* tests used in conventional analyses (such as statistical parametric mapping) are replaced with statistics from multivariate models to provide maps of the mutual information between locally distributed cortical responses and experimental variables (10). Here, questions about functional segregation have not changed fundamentally but are framed in terms of distributed neural computations at the voxel or subvoxel scale. We will see below that multivariate models (such as eigenimage analysis and dynamic causal modeling)

also play an important role in studying functional integration.

The Neurophysiological Basis of Imaging Signals

How many times have you read, “We know very little about the relationship between fMRI signals and their underlying neuronal causes”? In fact, decades of careful studies have clarified an enormous amount about the mapping between neuronal activity and hemodynamics (11–14). Furthermore, we know more than is sufficient to use fMRI for brain mapping. This is because the statistical models used to infer regionally specific responses make no assumptions about how neuronal responses are converted into measured signals (and that in particular do not assume this mapping is the same from voxel to voxel). In short, one does not need to know any neurophysiology to make precise inferences about where

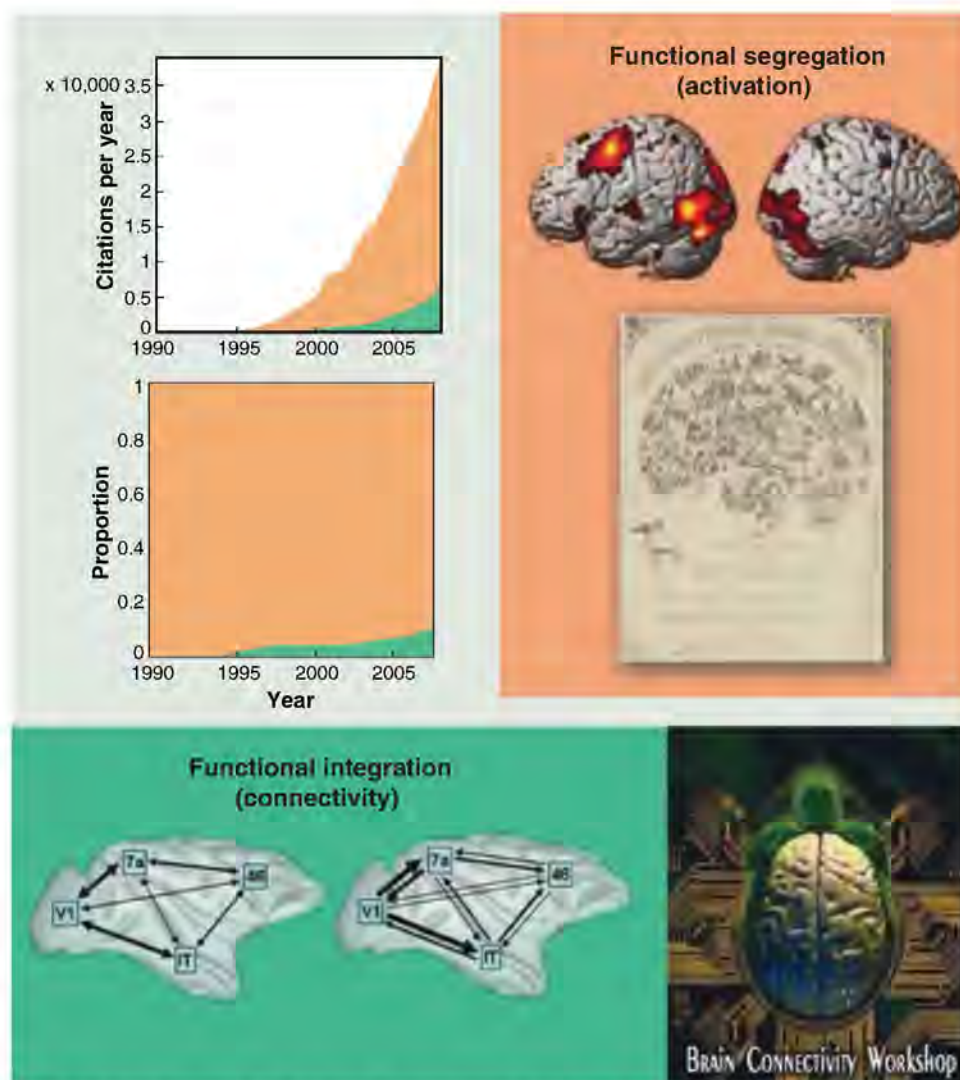


Fig. 2. Citation rates for the different applications of functional neuroimaging, derived in the same manner as for Fig. 1 but by searching for Activation AND functional imaging or Connectivity AND functional imaging with Topic = Neurosciences. This reflects the proportion of studies looking at functional segregation (activation) and those looking at integration (connectivity).

computations are taking place, provided there is some mapping between neuronal activity and hemodynamic responses (in a well-designed experiment, the only difference between conditions is neuronal activity, which is the only explanation for any hemodynamic difference).

So what are the questions asked when studying their relationship? These questions are about what can and cannot be inferred about neuronal activity from fMRI; for example, does fMRI reflect presynaptic inputs or postsynaptic firing (14); can it disambiguate between inhibitory and excitatory synaptic activity (13)? These questions address synaptic and microcircuit mechanisms in an attempt to disclose the relationship between spiking activity, local field potentials, and non-invasive imaging signals. Not only is this important for understanding what the brain is doing (in terms of local neuronal computations), it is critical for modeling distributed brain responses.

This is because fMRI responses do not cause each other; they are caused by hidden neuronal states, and one needs to understand the mapping from neuronal states to measured signals to make sensible inferences about effective connectivity (the influence that one neuronal system has on another). For example, in fMRI, there is a distinction between models of effective connectivity used by dynamic causal modeling and econometric models (such as structural equation modeling and Granger causality). In dynamic causal modeling, neuronal states are modeled explicitly, allowing for regional variations in the hemodynamic response function. This variation violates the assumptions of econometric models. Recent multimodal studies in rat models of epilepsy have shown these variations can have a profound effect on inferences about effective connectivity (15). Another important example is the relationship between hemodynamics and the

frequency of induced electrophysiological responses, in which higher frequencies (that may reflect neuromodulatory mechanisms) lead to increased fMRI signals (16).

Resting-State Correlations and Modes

There has been a recent upsurge in studies of fMRI signal correlations observed while the brain is at rest (17). These patterns seem to reflect anatomical connectivity (18, 19) and can be characterized in terms of remarkably reproducible patterns of functional connectivity called modes (20). One of these modes recapitulates the pattern of deactivations observed in activation studies (the default mode) (21). These studies have been received with much excitement (see <http://restingstate.stanford.edu/>) and some ambivalence: On the one hand, they are very interesting. They suggest that, even at rest, endogenous activity in the brain is self-organizing and highly structured. On the other, their relationship to neuronal dynamics (22) and the questions they pose (23) are not always clear. One view of resting-state correlations is that they forego hypothesis testing because they preclude experimental manipulations. So why are they so interesting? Perhaps because they address functional integration and distributed processing and do so in the context of structure-function relationships. In this context, there are many mechanistic questions about the genesis of autonomous dynamics and the structures that support them. Some of the most interesting work in this field has come from computational anatomy and neuroscience. The emerging picture is that endogenous fluctuations are a consequence of dynamics on anatomical connectivity structures with particular scale-invariant and small-world characteristics (24, 25). These are well-studied and universal characteristics of complex systems and suggest that we may be able to understand the brain in terms of universal phenomena. In short, endogenous fluctuations may be one way in which anatomy speaks to us through dynamics. Furthermore, they prompt important questions about how fluctuations shape evoked responses. In other words, are evoked brain responses and implicit computations affected by endogenous changes in its state (26)?

Some Interesting Failures

It is worthwhile to consider ideas that have not worked (and acknowledge those who tried to make them work). I will look at two examples, one from biophysics and one from neuroinformatics. A few years ago there was great excitement about the possibility of using fMRI to measure neuronal currents directly (27, 28). Despite some impressive scientific prospecting, the hope that we will be able to measure neuronal activity directly on a millisecond and millimeter scale throughout the brain is now receding. Basically, the sorts of signals caused by neuronal activity currently appear to be too small to measure. However, it is comfort-

ing to reflect that we can already measure, with exquisite temporal precision, neuronal currents using EEG and MEG.

In 2000, there was a bold experiment to see if fMRI data sharing would provide added value for the imaging community (29) in terms of methodological cross-validation and the opportunity to reanalyze data. Despite laudable efforts, the experiment failed; why? Functional neuroimaging is a leader in providing informatics models for standardization (such as data formats and anatomical spaces) and software sharing (such as data analysis packages). However, unlike genomic or astrophysical data, fMRI data per se are very context sensitive. This means if data are optimal for your question, they are suboptimal for mine. This somewhat subverts the *raison d'être* for sharing. Furthermore, on a lighter note, a reviewer observed that "it's much too easy to collect ... data (easier in fact than obtaining data from the data centre)."

Models and Questions

I have looked at recent developments in terms of the questions they address. The premise of this review is that the challenge for neuroimaging lies

in specifying the questions or competing models (hypotheses) that it can explore. The implicit equivalence between questions and models may explain why many recent advances in characterizing brain-imaging data rest on model comparison. We have been comparing models with classical inference from the inception of brain mapping (comparing null and alternative models about regionally specific brain activations). However, the range and nature of models we could compare is growing rapidly. The conceptual challenge ahead may not lie in finessing the techniques at our disposal but informing the models used to explain data. I will consider four examples of modelled neuroimaging, all of which engage with fields beyond neuroimaging.

Computational Neuroscience

Perhaps the most obvious place to look for well-posed questions or models is theoretical neurobiology and computational neuroscience. There are many compelling examples that use fMRI to adjudicate among models of neuronal computations and their functional architectures. These studies rest on replacing traditional explanatory

variables in statistical models of imaging data with quantities generated by computational models that are actually doing something. Nice examples here include the models of reinforcement and value learning (30–33). There is now an established tradition of taking a computational model that represents a hypothesis about how the brain evaluates contingencies, optimizing the model in relation to behavioral data, and using it to predict regionally specific fMRI responses (33). As a result of this approach, ventral striatal responses can now be treated as a proxy for unexpected rewards of the sort predicted by temporal difference models of learning. This is remarkable because a few years ago the only regionally specific correlates of reward came from invasive unit electrode recordings (34). Another nice example is in computational motor control, in which theories about optimal control can be evaluated against empirical fMRI data (35). The last example is the use of constructs from information theory to quantify novelty and surprise to see which parts of the brain encode causal regularities (or volatility) in our sensorium (36, 37).

Neuroeconomics

A pleasing example of synergy between imaging and another field is the use of constructs from behavioral economics. Neuroimaging is now a primary modality for the study of neuroeconomics and allows researchers to establish the neuronal infrastructures that may be responsible for encoding and computing things like expected utility, discounting, and other variables that shape our choices in social or economic interactions (38, 39). Another example, from the new field of social neuroscience, is the adoption of game theory to generate predictors of brain responses. In this context, model comparison allows one to lend a physiological validity to notions like guilt, regret, or altruism (40).

Biophysical Modeling

Model comparison also plays a key role in optimizing biophysical models. A pragmatic example here is the use of EEG data to adjudicate among forward models that map from sources in the brain to sensors. These models embody priors on the deployment of distributed neuronal sources and allow one to select the best prior assumptions. Put simply, one creates a number of models, each incorporating different but plausible assumptions (based on prior beliefs) about how data are generated. One then compares these models in terms of their evidence (the probability of obtaining data under that model). In some cases, this comparison is an implicit part of model optimization and can automatically switch off irrelevant neuronal sources (41). The same model comparison framework has proven extremely powerful in the comparison of dynamic causal models (42), in which hundreds of models (encoding different connectivity architectures) are

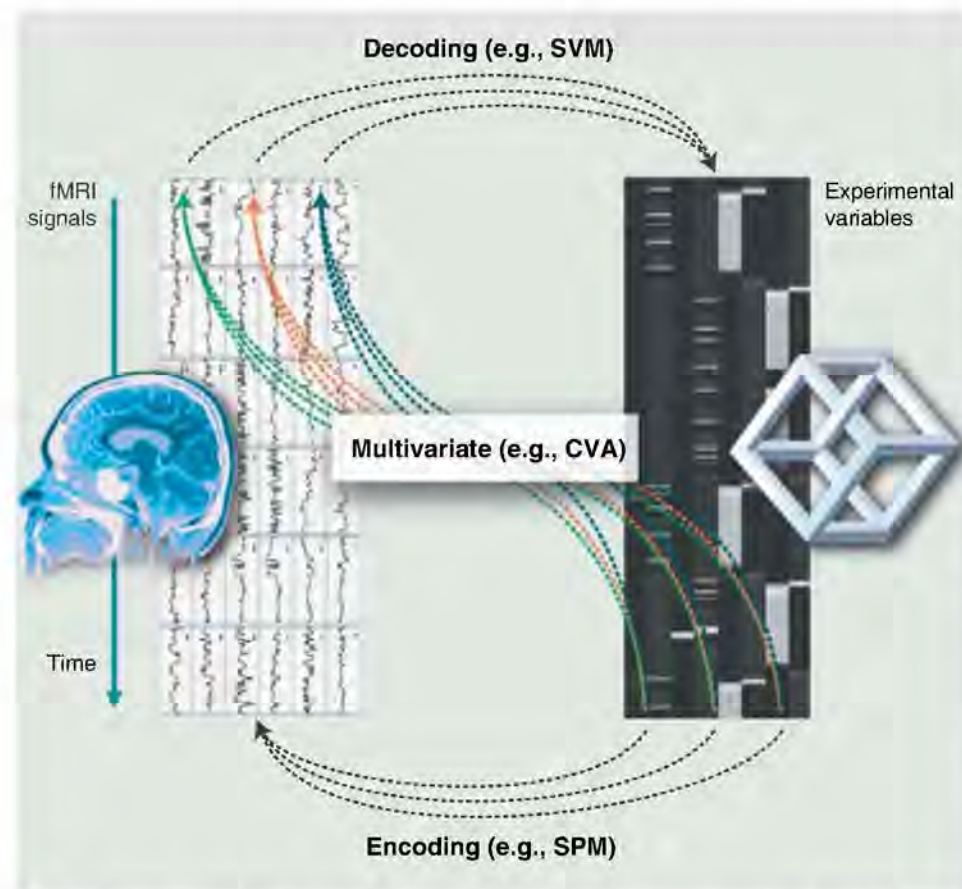


Fig. 3. Schematic showing the relationship between decoding (demonstrating a many-to-one mapping between activity in a local clique of voxels and an experimental variable), encoding (the conventional approach of showing a significant many-to-one mapping between experimental variables and activity in one voxel), and the more general multivariate many-to-many mapping between both sets of variables. SVM, support vector machine; SPM, statistical parametric mapping; CVA, canonical variates (or correlation) analysis.

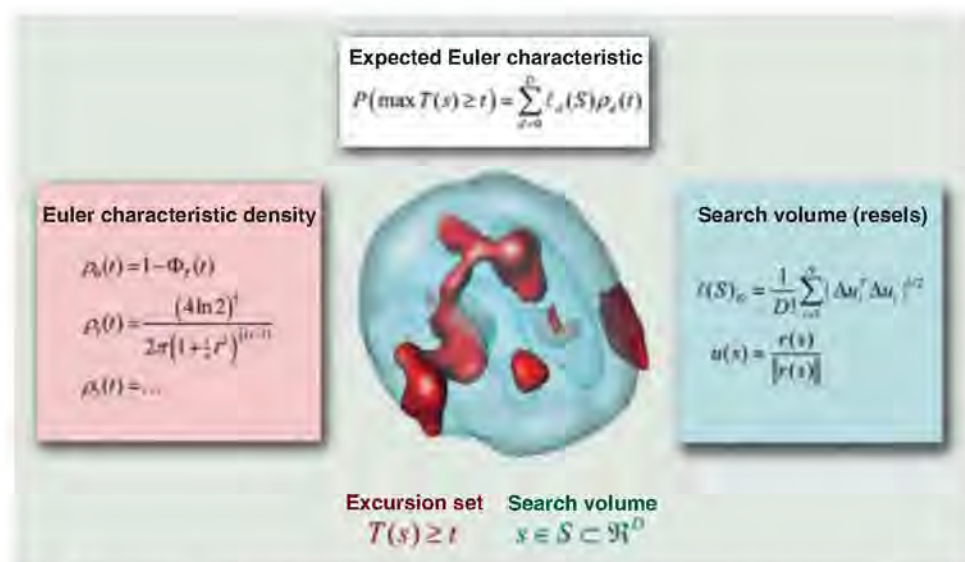


Fig. 4. Schematic showing some of the key equations behind topological inference. The basic idea is to split the problem of computing a P value (**top**) for a peak in a statistical image or map into two parts. The first part (**left**), the Euler characteristic density $\rho_d(t)$, depends only on the statistic and threshold t chosen for a D -dimensional search space; $s \in S$. This is the expected number of peaks per resolution element (resel). In this example, the equations pertain to the T statistic with a cumulative density Φ_T and ν degrees of freedom. The second part (**right**) is the search volume $\ell(S)_d$ measured in resels, which depends only on the shape and smoothness of the search space. This is defined in terms of the residuals $r(s)$ of the statistical test at each voxel; see (44) for details.

searched. These are an important class of models because many questions or hypotheses can be framed in terms of context-sensitive coupling between regions. Important questions here include the relative contribution of interhemispheric, bottom-up and top-down influences in cortical processing hierarchies and the implications for perception and action. These models have now been developed for fMRI, EEG, MEG, and local field potentials and are at the stage at which one can ask mechanistic questions about neuronal coupling by using, for example, animal models of Parkinson's disease (43).

Statistical Models

Imaging neuroscience has made some notable contributions to the physical sciences. In terms of statistical models for continuous data, neuroimaging has been at the forefront of developments, thanks to the contribution of people like Keith Worsley (who died prematurely a few months ago). Neuroimaging has essentially invented a new field of statistics, topological inference (based on random field theory), which underpins nearly all mainstream image analysis software (Fig. 4) (44). Similarly, some of the most advanced mathematical modeling of spatial data and their deformations has emerged in computational neuroanatomy (45). Finally, our systems models of connectivity and coupling are probably among the most developed in the biological sciences (46). These modeling contributions are not always heralded with the same applause as dis-

covery science but underpin model comparison and the ability to ask questions of our data.

Conclusion

In summary, the most promising avenues for the future may rest on developing better models of our data that complement and exploit the richness of these data. These models may well already exist in other disciplines (such as machine learning, machine vision, computational neuroscience, and behavioral economics) and may enable the broader neurosciences to access neuroimaging so that key questions can be addressed in a theoretically grounded fashion. When I started writing this review, I was looking for headline themes and imminent breakthroughs. However, these may be less important than the vast number of incremental studies that pervade and enrich nearly every corner of neuroscience. Perhaps what we should be celebrating 21 years later is the fact that any bright young student or seasoned researcher can engage with an imaging unit and start to ask questions they are passionate about.

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Direct Evidence for Spinal Cord Involvement in Placebo Analgesia

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Placebo analgesia is a prime example of how psychological factors can influence pain perception (1). It refers to a situation where the administration of an inactive treatment has a pain-relieving effect, presumably because of the participant's belief in the analgesic effectiveness of the treatment. Neurobiologically, placebo analgesia is in many cases opioid-dependent and relies on frontal cortical areas and their projections to downstream effectors in the brainstem (1, 2). One possible mechanism of placebo analgesia is thus that cortical areas recruit the opioidergic descending pain control system in the brainstem (3), which ultimately inhibits nociceptive processing in the dorsal horn of the spinal cord in a gate-control manner (4). Behavioral data support the idea that placebo analgesia can act at the level of the spinal cord (5), but there is no direct evidence that nociceptive responses in the spinal cord are reduced under placebo analgesia. We combined high-resolution functional magnetic resonance imaging (fMRI) of the human cervical spinal cord with a robust placebo analgesia paradigm (6) (fig. S1) to test the hypothesis that spinal cord blood oxygen level-dependent (BOLD) responses related to painful heat stimulation are reduced under placebo analgesia.

We first tested for the main effect of painful stimulation and observed the strongest BOLD responses in the dorsal horn ipsilateral to the side of painful stimulation at the expected segmental level [C6, approximately at the junction with C5; $t_{(12)} = 3.51$, $P = 0.002$; Fig. 1A]. Pain ratings, which were obtained after each stimulus during the fMRI exper-

iment, were significantly lower under the placebo condition as compared with the control condition [placebo rating of 52.3 ± 5.9 (mean $\pm$ SEM), control 71.1 ± 3.1 ; 26% reduction; $t_{(12)} = 3.56$, $P = 0.002$], indicating that our placebo induction was successful. We next tested whether the observed BOLD response in the ipsilateral dorsal horn (at the peak voxel of the main effect) would be decreased under the placebo condition. A reduction of BOLD responses under placebo compared with control was evident [$t_{(12)} = 1.81$, $P = 0.046$; Fig. 1B and fig. S2]. To further demonstrate the spatial specificity of our approach, we also tested for motor responses in a reaction time task [middle finger button presses (6)] and found these to be localized more inferiorly and anteriorly (segments C7 and C8; fig. S3), consistent with the functional neuroanatomy of the sensory-motor system.

Our data provide direct evidence that psychological factors can influence nociceptive processing at the earliest stage of the central nervous system, namely the dorsal horn of the spinal cord. They also reveal that one mechanism of placebo analgesia is inhibition of spinal cord nociceptive processing, possibly mediated by the descending pain control system (3) in a gate-control manner (4). It is likely that the decreased BOLD responses we observed are caused by endogenous opioids because opioid antagonists block placebo analgesia (1) and because recent fMRI data from rat spinal cord showed morphine depression of dorsal horn BOLD responses (7). However, our study cannot reveal the exact mechanism of spinal inhibition [i.e., effects on

primary afferents (presynaptic), interneurons, or projection neurons (postsynaptic)] and whether the observed effect is specific for nociception, because we did not measure responses to innocuous stimuli. Nevertheless, the demonstration that modulatory influences on nociceptive spinal cord activity are measurable by fMRI in humans opens up new avenues for assessing the efficacy and possible site of action of new treatments for various forms of pain, including chronic pain.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/326/5951/404/DC1

Materials and Methods

Figs. S1 to S3

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Fig. 1. Pain-related BOLD responses and their reduction by placebo. (A) (Left) The average structural image with the black box indicating the sagittal section (middle image) and the red line indicating the transverse section (right image). The sagittal and transverse sections show that BOLD responses (main effect of pain; visualization threshold $P < 0.01$ uncorrected) are present in the dorsal part of the spinal cord, ipsilateral to the

side of painful stimulation (left). The location corresponds to segment C6. The color bar indicates t values. (B) Parameter estimates were extracted from the peak voxel for the main effect of pain in the ipsilateral spinal cord. The parameter estimates show that the BOLD response is significantly reduced under placebo (gray bar) in comparison with control (white bar). Error bars indicate standard error; * $P \leq 0.05$.

Phase Transitions, Melting Dynamics, and Solid-State Diffusion in a Nano Test Tube

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Confined nanoscale geometry greatly influences physical transformations in materials. The electron microscope enables direct visualization of these changes. We examined the evolution of a germanium (Ge) nanowire attached to a gold (Au) nanocrystal as it was heated to 900°C. The application of a carbon shell prevented changes in volume and interfacial area during the heating cycle. Au/Ge eutectic formation was visualized, occurring 15°C below the bulk eutectic temperature. Capillary pressure pushed the melt into the cylindrical neck of the nanowire, and Ge crystallized in the spherical tip of the carbon shell. Solid-state diffusion down the length of the confined Ge nanowire was observed at temperatures above 700°C; Au diffusion was several orders of magnitude slower than in a bulk Ge crystal.

Transmission electron microscopy (TEM) can enable the visualization of nanoscale systems undergoing physical transformations. For example, studies of nanocrystal and nanowire melting, growth, and coalescence have provided new insight about how scale and interfaces affect phase behavior (1–5). Dynamic processes, such as thermal expansion (6) and flow (7, 8), have been observed in real time by TEM, and studies of more complicated chemical mixtures, such as two-component systems, have yielded insight about phase nucleation and crystallization (8–11). Nonetheless, it remains a challenge to manipulate individual nanostructures in a TEM and to probe physical changes while varying environmental conditions without destroying the nanostructure. For instance, the nanoscale systems in previous studies have been free to change shape, thus adding two additional thermodynamic variables—volume and surface area—to the experiment. Ultimately, the nanostructures themselves are destroyed, or at the very least changed substantially in size or shape, when a phase change, such as melting, occurs.

In this context, we used a volume-restricting carbon shell as a “test tube” to conduct a thermodynamic experiment on a nanoscale system. We deposited a thin, rigid carbon layer around the tip of a germanium (Ge) nanowire with an attached gold (Au) nanocrystal in a TEM and imaged it as it was heated to 900°C (12). With this approach, the eutectic temperature and liquidus composition could be measured without a loss of the nanostructure or substantial changes in volume throughout the heating cycle.

The Ge nanowire with attached Au seed in Fig. 1A was imaged as the temperature was

increased steadily at 10°C/min (movies S1 to S3). Carbon was deposited on the nanowire at a rate of 2 nm/min, reaching a final thickness of 140 nm at 675°C. Carbon shell and filament formation by electron irradiation in the TEM is well known to occur when carbon is present, as in this case with Ge nanowires coated with a monolayer of 1-hexene (4, 5, 13). At room temperature, an abrupt interface exists between the Au particle and Ge nanowire, and a thin Ge shell surrounds the Au seed (14). As the temperature increased, the Au particle dissolved the Ge shell and expanded slightly.

When the temperature reached 346°C, the seed particle melted to form a Au/Ge eutectic mixture (Fig. 2 and movie S1). There was a volume increase of 45%, consistent with an increased Ge solubility from 3 to 28%. The volume expansion pushed the seed partially into the neck of the nanowire. The slight depression of the eutectic temperature results from the limited size of the seed particle and nanowire and is consistent

with expectations [supporting online material (SOM) text] (15–17).

As the temperature was raised above the eutectic point, the melt shifted into the cylindrical neck of the nanowire and solid Ge recrystallized in the spherical tip (movie S1). There is a capillary pressure p_c , due to the difference in Laplace pressures ΔP , in the spherical cap and the cylindrical neck of the nanowire (18)

$$p_c = \Delta P_{\text{sphere}} - \Delta P_{\text{cylinder}} = \gamma \left[\left(\frac{2}{r_{\text{sphere}}} \right) - \left(\frac{1}{r_{\text{cylinder}}} \right) \right] \quad (1)$$

γ is the surface energy (units of energy per area) of the melt and r is the radius (table S1). The capillary pressure is 11 MPa in the direction of the cylindrical neck (SOM text). Without a confining carbon shell, the melt droplet would simply grow and dissolve more Ge as has been observed in previous studies (11, 19).

Ge recrystallization in the spherical bulb occurs by a process called metal-induced crystallization (20), which in this case is induced by the capillary pressure in the system. Additionally, the Au/Ge melt movement out of the bulb is not steady (Fig. 3C and movie S2). Most likely, the polycrystallinity of the Ge bulb gives rise to this motion: One Ge grain crystallizes and then stops, until another crystalline grain overcomes the nucleation barrier and grows.

At higher temperature (>400°C), the Au/Ge melt expanded even further into the nanowire, as shown in Fig. 4, dissolving more Ge. Figure 3A plots the measured Au/Ge melt composition in comparison with the bulk liquidus composition (21, 22). The liquidus composition in the nanowire was only slightly more concentrated in Au than expected from the bulk phase diagram, but this small deviation could be a kinetic effect related to the stepwise dissolution of the Ge crystal. In contrast, Sutter *et al.* (11) found that Au/Ge droplets at the end of Ge nanowires

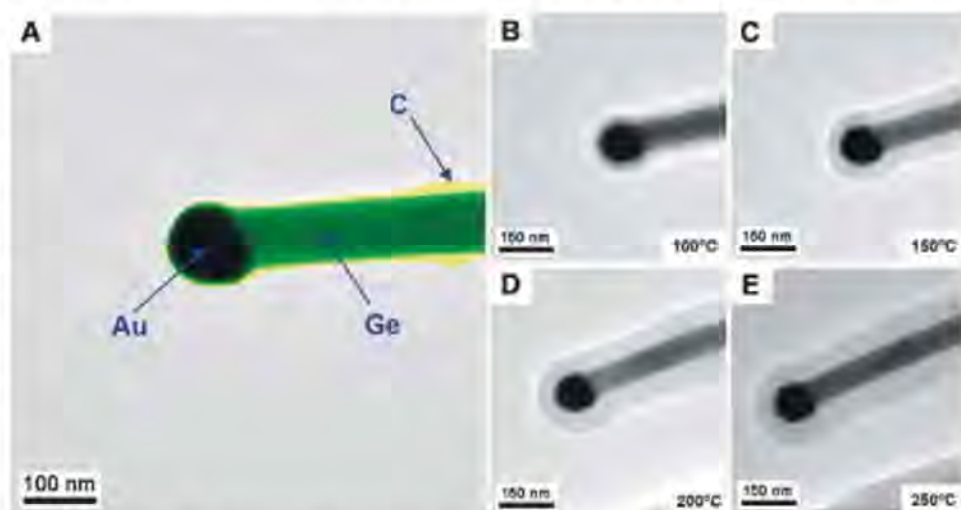


Fig. 1. (A to E) TEM images of a Ge nanowire (75 nm in diameter) with a Au nanocrystal (100 nm in diameter) at its tip. (B) to (E) The thin carbon layer [highlighted in yellow in (A)] increased in thickness as the nanowire was heated.

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Fig. 2. (A to M) TEM images of the Au seed particle as it melted at 346°C and migrated into the stem of the nanowire with increased temperature. Ge recrystallized in the spherical end as the Au/Ge melt shifted into the neck of the nanowire (movie S1).

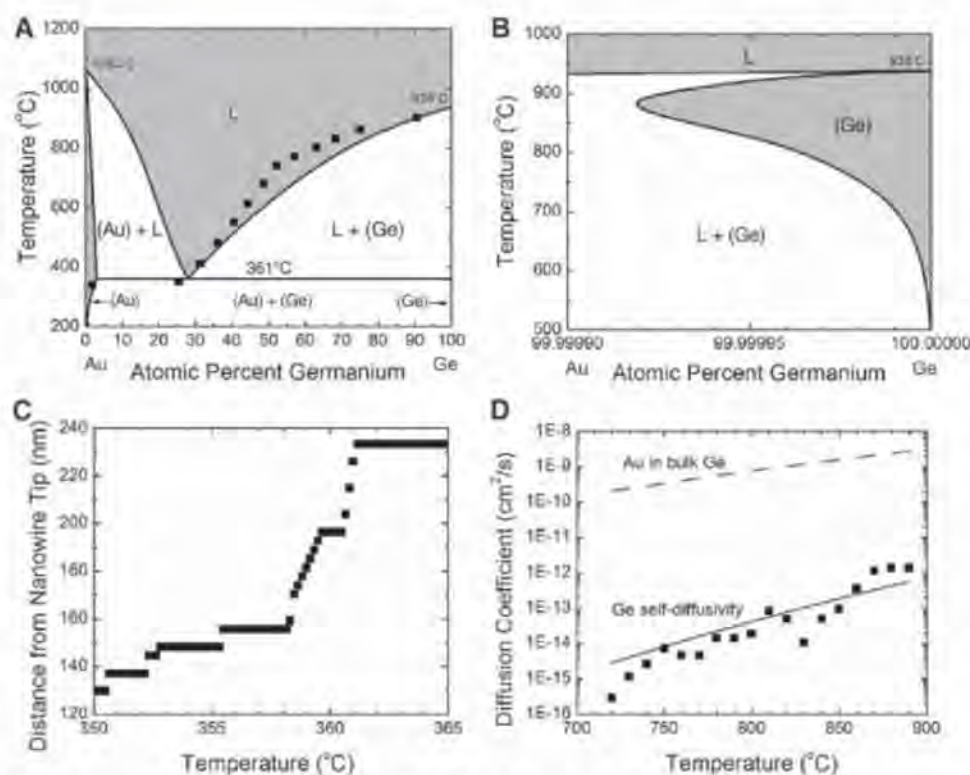
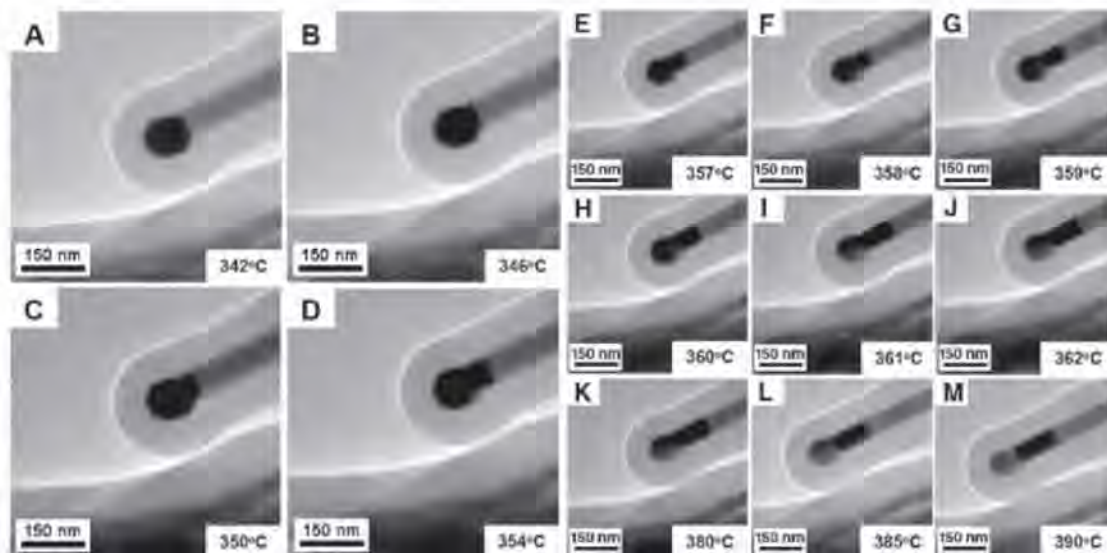


Fig. 3. (A and B) Au/Ge phase diagrams (21) with the observed Au/Ge melt compositions (squares) shown in (A). (C) Position of the liquid/solid interface plotted against the temperature of the system. There is stepwise discontinuous motion of the liquid/solid interface as additional Ge is dissolved and recrystallized. (D) The measured solid Au-in-Ge diffusion coefficient (squares) plotted as a function of temperature compared to the diffusivity of Au in bulk Ge (dashed line) and Ge self-diffusivity (solid line).

became much richer in Ge than in the bulk when heated above the eutectic temperature. In their study, however, the volume of the Au/Ge drop was unrestricted and grew as the temperature increased. The increased droplet size reduces the surface curvature and the corresponding energy density of the melt (23) and consequently causes more Ge to dissolve than the equilibrium concentration in the bulk (19). The fixed surface curvature provided by the carbon shell prevents such changes in energy density, apart from the initial shifting of the melt from the spherical cap to the cylindrical neck of the nanowire.

At 800°C, a band of material in front of the melt/solid interface became visible (Fig. 4). The solid solubility of Au in Ge increases rather substantially in this temperature range (Fig. 3B), and diffusion lengths become long enough to be observed (24). The leading edge of the band advances smoothly, but the melt stops and starts, periodically catching up with and consuming portions of the band before more diffusion occurs (movie S3). The images enabled an estimation of the diffusion coefficient, which ranged from 10^{-16} cm²/s (at 700°C) to 10^{-12} cm²/s (at 900°C) (25). Figure 3D shows the observed diffusion

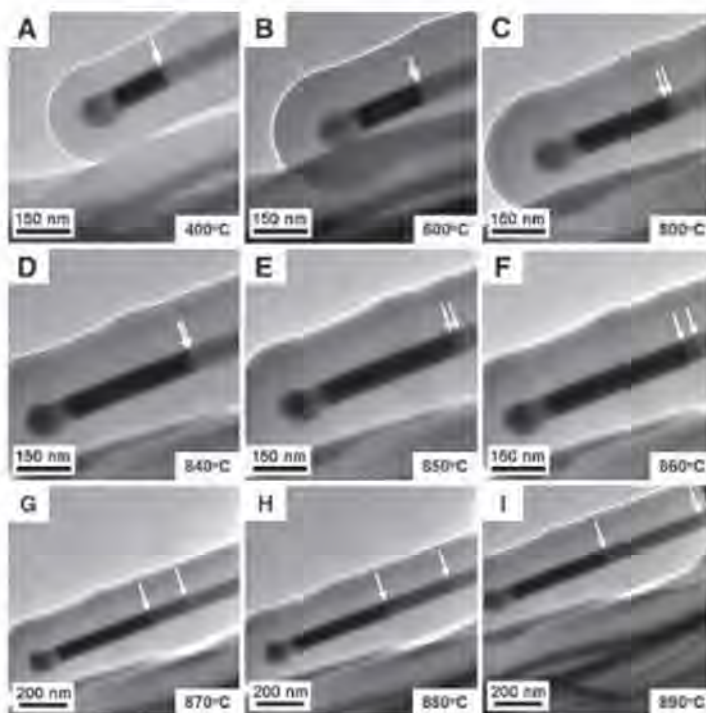
coefficient (D) as a function of temperature (T): $D = D_0 \exp(-E_A/k_B T)$ (where E_A is the activation energy and k_B is the Boltzmann constant), with $D_0 = 10^{7.2}$ cm²/s and $E_A = 4.3 \pm 0.3$ eV. These values are several orders of magnitude lower than the values for the diffusion of Au in bulk Ge, which range between 10^{-10} cm²/s (700°C) and 10^{-8} cm²/s (900°C), with corresponding $D_0 = 10^{-2}$ cm²/s and $E_A = 1.5$ eV (26).

Au is apparently diffusing by a different pathway in the confined nanowires than in a bulk crystal of Ge. Au exists predominantly as a substitutional impurity in Ge, but a very small number of Au atoms (~ 1 in 10^5) exist as interstitials, created by interstitial-substitutional exchange (27–29). These interstitial species are highly mobile, with diffusivities typically on the order of $D_i \approx 10^{-5}$ cm²/s at 700°C (27). Au atoms can also reconfigure to form charged interstitial-vacancy pairs (27–29). The pairs can move through the solid as well; when a Au atom forms an interstitial-vacancy pair, an adjacent Ge atom can be accepted by the transient vacancy, effectively causing the Au and Ge atoms to switch positions (27–29). Au diffuses by a combination of these two mechanisms, and the diffusivity depends on the intrinsic diffusivity of the free interstitial Au atoms D_i , the bound interstitial-vacancy pairs D_{Au-V} , and the relative fractions of interstitial Au atoms χ_i and interstitial-vacancy pairs χ_{Au-V} , present within the total Au population (27–29)

$$D_{Au-Ge} = \chi_i \times D_i + \chi_{Au-V} \times D_{Au-V} \quad (2)$$

In bulk Ge, the diffusion coefficient at 700°C is $D_{Au-Ge} = 10^{-10}$ cm²/s (26), implying that it is the small fraction ($\chi_i \approx 10^{-5}$) of highly mobile ($D_i \approx 10^{-5}$ cm²/s) free Au interstitials that control the rate of diffusion in bulk samples; that is, $D_{Au-Ge} \approx \chi_i \times D_i$. In contrast, the small scale of the nanowire system virtually eliminates any contribution from isolated Au interstitials. Only 10^8 Au atoms are present in the entire nanowire, and solubility limits constrain the maximum

Fig. 4. (A to I) TEM images of the Au/Ge melt as it expanded up the shaft of the nanowire with increased temperature (movie S3). The light gray band between the arrows shows Au diffusing up the solid Ge wire. Images (A) to (F) are magnified 1.5 times more than images (G) to (I).



number of Au atoms present in the crystalline Ge stem to 10^4 (30). Because only 1 in every 10^5 Au atoms exists as an interstitial impurity, the limited number of Au atoms in the nanowire ensures that free interstitial Au atoms are nearly nonexistent. Consequently, bulk diffusion rates are not observed. Instead, the rate of diffusion in the nanowire is controlled by Ge atoms recombining with adjacent $\text{Au}_i\text{-V}$ pairs, resulting in $D_{\text{Au-Ge}} \approx \chi_{\text{Au-Ge}} \times D_{\text{Au}_i\text{-V}}$ with diffusion coefficients comparable to that of Ge self-diffusion.

It is worth mentioning that the observed diffusion coefficients also agree well with those of Ge self-diffusion, which have values ranging from $10^{-15} \text{ cm}^2/\text{s}$ at 700°C to $10^{-12} \text{ cm}^2/\text{s}$ at 900°C ($D_0 = 13.6 \text{ cm}^2/\text{s}$; $E_A = 3.1 \text{ eV}$). However, Ge self-diffusion occurs with isolated vacancies acting as acceptors for adjacent Ge atoms (31)—a mechanism that is difficult to rationalize in the nanowire case. The intrinsic vacancy concentration in Ge at 800°C is 10^{13} cm^{-3} , or roughly 10 vacancies for every cubic micrometer of crystal (32); a Ge nanowire 75 nm in diameter with a length of $25 \mu\text{m}$ has only a single vacancy, which is surely not sufficient to sustain diffusion to any appreciable extent.

The interface of a Ge nanowire attached to a Au seed particle was observed by TEM as it was heated to 900°C within a volume-restricting carbon shell. The Au seed particle melted when the temperature reached the eutectic point, which was slightly depressed relative to the bulk. The melt then shifted from the spherical cap to the neck of the nanowire, and Ge recrystallized in its place. This movement was the result of a capillary pressure within the carbon shell. At higher temperature, solid-state diffusion of Au into the Ge nanowire was observed, revealing that the rate-limiting mechanism for Au diffusion in the nano-

wire is different from that in the bulk. Defect concentrations in nanowires are much different than in the bulk crystal, and in this case give rise to a fundamentally different rate process. In bulk Ge, free interstitial Au atoms are the dominant diffusing species; however, in the nanowire, Au atoms appear to diffuse predominantly as bound $\text{Au}_i\text{-V}$ pairs, leading to diffusion rates that are orders of magnitude slower. Stable confinement of the nanoscale Au-Ge system by the carbon shell enabled the observation of a variety of physical transformations without a loss of the nanostructure or a change in size and shape, providing a picture of how size and shape affect these physical processes.

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18. For a cylindrical capillary, the meniscus is a hemispherical cap with capillary pressure $2\gamma\cos\theta/r$. The spherical bulb at the tip of the nanowire has a capillary pressure of $2\gamma/r$. The pressure drop arises because the capillary pressure in the cylinder is γ/r .
19. Imagine a free-standing liquid Au/Ge droplet at a given temperature with a Ge concentration equal to the bulk equilibrium value. This system is not in equilibrium because of the surface curvature of the droplet (the Kelvin effect). The droplet can reduce this surface curvature, and corresponding energy density, by growing. If there were large sinks of both Au and Ge available, then the droplet would dissolve more Ge and Au to grow in size, while maintaining the bulk equilibrium ratio of Au to Ge. When the liquid droplet is interfaced with a Ge nanowire, there is no additional Au available to melt, and therefore the droplet can only grow and reduce its energy density by melting additional Ge to increase in size. This results in a higher Ge concentration than the bulk equilibrium value at that temperature.
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22. The Au/Ge melt composition χ_{Ge} was determined by accounting for the amount of Ge dissolved from the nanowire into the melt $\chi_{\text{Ge}} = \frac{(V_{\text{Ge}}^{\text{melted}} - V_{\text{Ge}}^{\text{recrystallized}})\rho_{\text{Ge}}}{V_{\text{Au}}\rho_{\text{Au}} + (V_{\text{Ge}}^{\text{melted}} - V_{\text{Ge}}^{\text{recrystallized}})\rho_{\text{Ge}}}$. ρ_{Ge} and ρ_{Au} are the atomic densities of Ge and Au, V_{Au} is the initial Au seed volume, and $V_{\text{Ge}}^{\text{melted}}$ and $V_{\text{Ge}}^{\text{recrystallized}}$ are the volumes of Ge melted from the stem and recrystallized into the bulb, respectively (movie S1).
23. Energy density (J/m^3) is equivalent to pressure (N/m^2). The surface energy per unit of area γ (J/m^2) does not depend on the dimensions of the system, and the total surface energy $A \times \gamma$ actually increases with increasing droplet size. The important term is $\partial A/\partial V$, where the energy density (or pressure) is given by the Young-Laplace equation $\Delta P = \gamma(\frac{1}{R_1} + \frac{1}{R_2}) = \gamma(\frac{2}{R})$.
24. A Au-substituted Ge phase is not observed below 800°C because the diffusion length is too short to observe. At low temperatures, Au can only diffuse into the Ge nanowire a few nanometers past the melt/solid interface before being incorporated into the advancing Au/Ge melt shortly thereafter. For example, after 1 min at 700°C , Au can only diffuse about 2 nm into the Ge crystal; at 800°C , the diffusion length is closer to 20 nm.
25. At T (at some time t), the initial interface location x_i was marked at time $t - \frac{1}{2}dt$, and again the final interface location x_f was marked at time $t + \frac{1}{2}dt$. The average diffusion coefficient was then calculated from the diffusion length $x = 2\sqrt{Dt}$ for that particular temperature, as $D = (x_f - x_i)^2/4dt$.
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Supporting Online Material

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Materials and Methods

SOM Text

Table S1

Movies S1 to S3

References

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The Packing of Granular Polymer Chains

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Rigid particles pack into structures, such as sand dunes on the beach, whose overall stability is determined by the average number of contacts between particles. However, when packing spatially extended objects with flexible shapes, additional concepts must be invoked to understand the stability of the resulting structure. Here, we examine the disordered packing of chains constructed out of flexibly connected hard spheres. Using x-ray tomography, we find that long chains pack into a low-density structure whose mechanical rigidity is mainly provided by the backbone. On compaction, randomly oriented, semi-rigid loops form along the chain, and the packing of chains can be understood as the jamming of these elements. Finally, we uncover close similarities between the packing of chains and the glass transition in polymers.

It is an enduring puzzle why a boxful of ball bearings, even when compacted by many taps, never packs denser than ≈ 0.64 (1–6). This is much less dense than Kepler's stacking of triangular layers, known to every grocer as the optimal packing of oranges (packing density ≈ 0.74). Yet despite their suboptimal density and lack of periodic order, jammed packings are rigid and resist shear. Replacing spheres with less symmetric objects (such as rods or ellipsoids) introduces new degrees of freedom that alter the packing structure and create new modes of response (7–12). Here, we describe the jammed packing of flexible granular chains. Although density and coordination number decrease dramatically with increasing chain length, the packings remain rigid. Using x-ray tomography to visualize the chain conformations, we find that long floppy chains effectively partition into a collection of nearly rigid elements—small loops—that then jam into a rigid packing. Randomly packed spheres have often been used as a model for simple glasses (2, 13). Building on this result and also on the fact that polymer molecules are often modeled as flexible chains (14, 15), we find that the jammed packing of chains captures the dependence of the polymer glass transition on chain length, topology, and stiffness.

Our chains are the familiar ball-chains commonly used as window shade pulls (Fig. 1B, inset image): The “monomers” are hollow metal spheres, and the “bonds” are short metal rods. The monomers are uniform in size and evenly distributed along the chain; they are also free to rotate about the backbone so that the chain does not support torsion. These ball-chains are not arbitrarily flexible: There is a maximum bond-flex angle $\theta_{\max}$. We use the minimum loop size $\xi = 2\pi/\theta_{\max}$ as a simple measure of chain stiffness; this is the minimum length of chain that can close to form a ring. We used an aluminum chain with monomer diameter $a = 2.4$ mm and $\xi = 7.5$, as well as a brass chain with $a =$

1.9 mm and $\xi = 11.2$. For each, we studied the packing of linear chains with free ends and of cyclic chains whose ends are connected together in a loop.

The chains are loaded into a cylindrical cell mounted on an electro-mechanical shaker: Long chains are rapidly unspooled into the cell end-first; short chains are poured in a few at a time. The chains are compacted by giving the cell discrete vertical “taps”: a single period of 30-Hz sinusoidal vibration with peak-to-peak acceleration of 8g, where $g = 9.8$ m/s². After each tap, the pack height is measured to find the packing density ρ . The compaction dynamics of chains is similar to that of hard spheres (6): ρ increases in a logarithmic fashion with the number of taps and is slow to approach a steady state; we chose 10^4 taps as an arbitrary stopping point to measure the “final” density ρ_f . The packing produced is always disordered with no signs of chain crystallization. We repeated these measurements using cells of two different diameters (4.75 and 2.5 cm); this had no strong effect on our results.

Figure 1A plots ρ_f versus M , which is the number of monomers per chain. For linear chains, ρ_f falls monotonically with increasing M , from $\rho_f \approx 0.64$ for $M = 1$ to a much-reduced asymptotic value $\rho_{f,\infty}$ for the longest chains ($M \rightarrow \infty$). The asymptotic density is slightly higher for the floppy chain

($\xi = 7.5$ and $\rho_{f,\infty} = 0.43$) than for the stiff chain ($\xi = 11.2$ and $\rho_{f,\infty} = 0.39$). For linear chains, variation in M changes the density of chain ends in the pack. For cyclic chains, end effects are absent. As expected, long cyclic and linear chains pack at the same asymptotic density; however, short (but still floppy) cyclic chains ($M \geq 2\xi$) also packs at $\rho_{f,\infty}$. Still smaller ($M \rightarrow \xi$), semi-rigid cyclic chains actually pack less densely than the long chain limit. The trend in cyclic chains is opposite that found in linear chains, where short chains pack denser than long ones.

The observation that small, floppy cyclic chains pack at $\rho_{f,\infty}$ suggests that end effects are responsible for the enhanced packing density of short linear chains (in the floppy regime). We can check this directly by packing a mixture of linear and cyclic chains of the same length. As shown in Fig. 1B, ρ_f increases linearly with the fraction of linear chains. This indicates that in a packing of floppy chains, ends act as non-interacting, density-enhancing defects.

To reveal the detailed packing structure, we used x-ray tomography. Only the $\xi = 7.5$ Al chain is sufficiently x-ray transparent to be imaged. Our x-ray source is a 37-keV beam (GSECARS beam line) at the Argonne Advanced Photon Source. The beam size limits us to a relatively small sample: The chains are confined inside a cylinder of diameter 2.5 cm (≈ 10.5 monomer diameters), and the imaging volume covers 5 cm (out of ≈ 20 cm) of the packing column, containing 1000 to 1500 monomers. On the other hand, the high image resolution (27.5 μ m per voxel) allows us to accurately locate every monomer and trace every bond.

Figure 2A shows the pair distribution function $g(r)$ for packings of linear chains from $M = 1$ to 4096. The most notable change with M is in the structure of the second peak, associated with second-nearest neighbors. For spheres ($M = 1$), the second peak of $g(r)$ is split into two subpeaks, a well-known feature corresponding to two distinct particle arrangements (16). The subpeak at $r/a = 2$ corresponds to a linear trimer; for chains, this configuration naturally suggests three successive monomers along a backbone. The subpeak

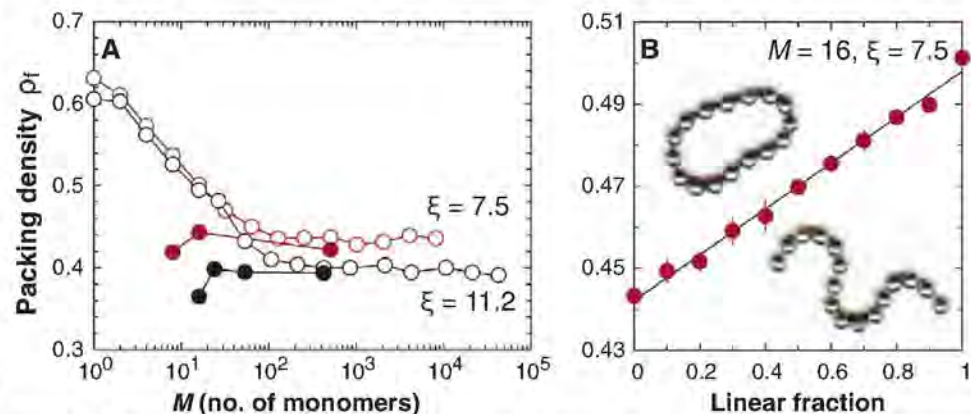


Fig. 1. (A) Packing density ρ_f of ball-chains versus chain length M after 10^4 taps, for both linear (open circles) and cyclic (solid circles) chains. Each data point is the average of five trials; error bars are smaller than the size of the symbols. (B) Packing density of a mixture of linear and cyclic chains (both with $M = 16$) as a function of the fraction of linear chains. Solid line is a linear fit.

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at $r/a = \sqrt{3}$ corresponds to a rhomboid cluster of four particles (Fig. 2A, inset); for chains, this requires contact between monomers without a shared bond. As we move to long chains, the $r/a = \sqrt{3}$ subpeak quickly disappears, indicating a sharp suppression in the number of contacts between monomers that do not share a bond. For spheres, the mechanical stability of the packing is provided by pair-wise contacts. For long chains, the suppression of pair-wise contacts between monomers that do not share a bond leaves the backbone as the remaining source of rigidity. But how can floppy chains be arranged into a rigid structure?

Unlike the $r/a = \sqrt{3}$ subpeak, the subpeak at $r/a = 2$ persists as M increases: It broadens and shifts slightly to $r < 2a$. This shift suggests that a large number of bonds must be flexed; for $M = 4096$, the peak center at $r/a = 1.89$ corresponds to a flex angle of 38° (Fig. 2B, inset). Measured directly, the flex angle distribution $p(\theta)$ for long chains ($M = 4096$) peaks strongly at $\theta_{\max} = 48^\circ$; in contrast, $p(\theta)$ for short chains ($M = 8$) is much flatter (Fig. 2C). We also compute the bond-orientational correlation $C_b(s - s')$ along a single chain

$$C_b(s - s') = \langle \vec{b}(s) \cdot \vec{b}(s') \rangle_s \quad (1)$$

Here, s and s' are monomer labels, and $\vec{b}(s)$ is the bond connecting monomers s and $s + 1$. Figure 2D shows $C_b(s - s')$ for compacted long chains. It decays rapidly from unity and becomes maximum anticorrelated at $s - s' \approx 6$ before returning to zero and executing small amplitude

oscillations (period $\approx 10.5a$). This observation indicates that there is no long-range orientational order along the backbone; instead, the local chain conformations contain a prevalence of near-minimal, semi-rigid loops (recall $\xi = 7.5$) (Fig. 3). For comparison, the $g(r)$ for $M = 8$ cyclic chains, which are rigid loops, is very similar to that for long chains, save for subtle differences: The second peak is sharper and is located at $r/a = 1.85$ (rather than 1.89), corresponding to next-nearest vertices of an octagon. The $g(r)$ of the $M = 16$ cyclic chain is nearly indistinguishable from that for long chains (Fig. 2B).

We suggest that these strongly flexed bonds and near-minimal loops are responsible for the rigidity of long-chain packings. Specifically, rigidity arises from the jamming of rigid elements (loops) that “condense” out of a floppy object (the chain) upon compaction. Consider a pack of minimal loops, each with ξ monomers; these will be completely rigid rings. A ring has six degrees of freedom; for a pack of rings to be jammed (i.e., mechanically rigid), there must be on average six independent constraints per ring. These are provided by pair-wise contacts between rings. On jamming, each ring is on average in contact with 12 other rings; on a per-monomer basis, each monomer will have, on average, $12/\xi$ contacts or $z = 2 + 12/\xi$ nearest neighbors (coordination number). Compared with frictionless spheres, where the mean coordination at jamming is $z = 6$, a jammed pack of rigid rings is much less coordinated and therefore less densely packed. Because $z \sim 1/\xi$, the packing density will be even lower for stiffer chains (larger ξ).

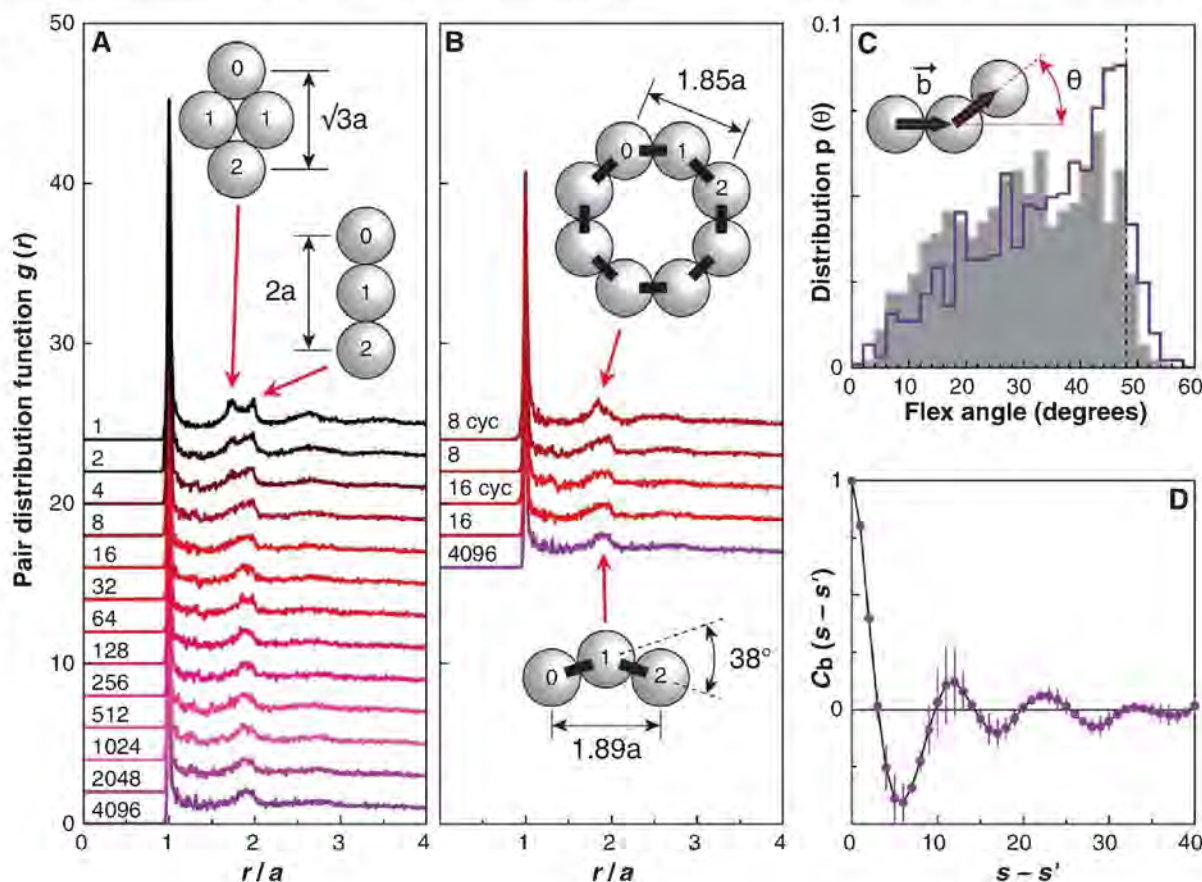
The loops in compacted chains are nearly, but not exactly, minimal in size; they are semi-rigid and have more degrees of freedom than do rigid rings. In addition, not all bonds are maximally flexed, and not all monomers belong on a loop. For linear chains, the chain ends, being bonded to only a single partner, are less constrained than monomers in the middle of a backbone. Each of these effects increases the number of constraints needed before a pack can jam, thereby enhancing the mean coordination and packing density at jamming. Therefore, we expect the following: (i) Long linear chains should pack less densely than short linear chains, but small, (nearly) rigid cyclic chains should pack the least densely of all. (ii) Stiff chains with large loop sizes should pack less densely than floppy chains with small ξ . Qualitatively, these statements agree with our experimental observations.

In the spirit of the jamming phase diagram (17), which links the glass transition of simple glass-formers with the jamming of grains, we suggest there is a similar connection between polymers and macroscopic chains. One well-studied aspect of the polymer glass transition is the variation of the glass transition temperature T_g on chain length and chain topology. For linear polymers, as $M \rightarrow \infty$, T_g quickly asymptotes to a constant value; whereas T_g decreases rapidly as $M \rightarrow 1$. This is commonly described by the Flory form

$$T_g = T_{g,\infty} - K/M \quad (2)$$

where $K > 0$ is a polymer-specific parameter (18). For cyclic polymers, in the long-chain limit, T_g is

Fig. 2. The pair distribution function $g(r)$ for compacted packing of chains ($\xi = 7.5$); curves are shifted for clarity. (A) Linear chains, from $M = 1$ to 4096. (B) Short cyclic (cyc) chains, compared with linear chains of the same length and also with a long linear chain. (C) The distribution of bond-flex angles for long ($M = 4096$, solid purple outline) and short ($M = 8$, gray shaded area) chain packings; the dashed vertical line indicates $\theta_{\max}$. (D) The bond-orientational correlation $C_b(s - s')$ measured along a single chain, averaged over three long chain packings ($M = 1024$, 2048, and 4096).



the same as that of long linear polymers of the same type, but as M becomes very small, T_g increases (19). As with ball-chains, the behavior of linear and cyclic polymers follows opposite trends as M becomes small.

The jamming phase diagram suggests a way to directly compare glassy polymers with jammed chains. Here, temperature T and specific packing volume $v \equiv 1/\rho$ are orthogonal axes; jammed/glassy states occupy the high-density, low-temperature region of the phase diagram, and fluid states occupy the rest. The glass transition is the transition from liquid to glass along the T axis, whereas jamming is the transition from unjammed to jammed states along the v axis at $T = 0$ (17). In this sense, $T_g(M)$ and $v_f(M) \equiv 1/\rho_f(M)$ should be analogous quantities. When we compare $T_g(M)/T_{g,\infty}$ for linear and cyclic poly(dimethylsiloxane) (PDMS) and $v_f(M)/v_{f,\infty}$ for the packing of chains, we see that there is a marked similarity between the two (Fig. 4).

As shown earlier, in a packing of floppy linear chains, chain ends act as non-interacting, density-enhancing defects. We can then write the specific packing volume v_f in the floppy regime as the weighted sum of v_{es} contributed by ends, and $v_b > v_{es}$ due to the bulk

$$v_f(M) = \left(1 - \frac{2l}{M}\right)v_b + \left(\frac{2l}{M}\right)v_{es} \\ = v_b - \frac{2l(v_b - v_{es})}{M} \quad (3)$$

Here $l \sim \xi$ is the extent that end influences propagate into the bulk of the chain. Cast in this

Fig. 3. Tomographic reconstruction of a packing that consists of a single long chain ($M=4096$). (A) The full reconstructed three-dimensional image. (B) A small slice of the packing, indicated by the shaded box in (A), projected onto the horizontal plane. The brighter a particle is, the closer it is to the center plane of the slice. Highlighted particles are arranged in three near-minimal loops.

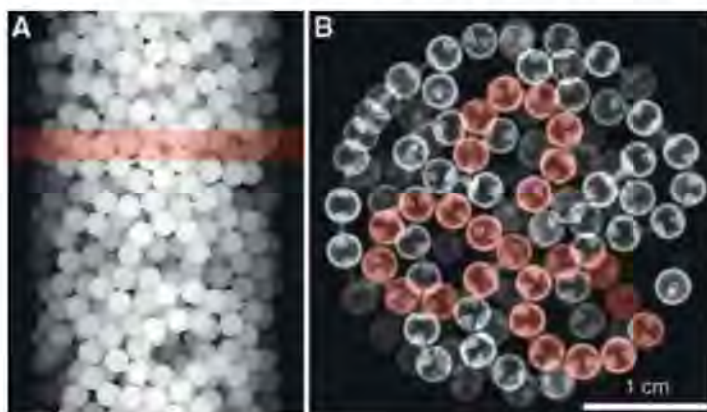
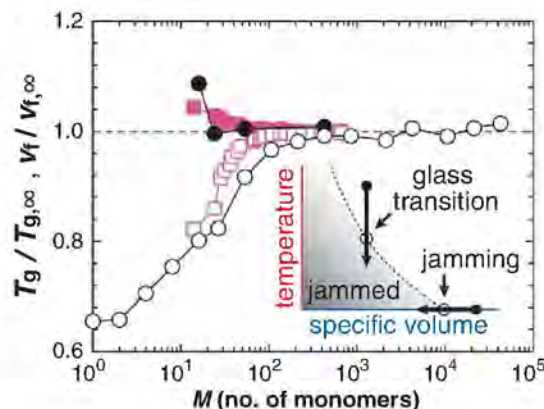


Fig. 4. The glass transition temperature $T_g(M)$ of linear and cyclic PDMS (open and solid squares) compared with the specific packing volume $v_f(M) \equiv 1/\rho_f(M)$ of linear and cyclic $\xi = 11.2$ ball-chains (open and solid circles). Both are normalized by their asymptotic $M \rightarrow \infty$ values $T_{g,\infty}$ and $v_{f,\infty}$. PDMS data are taken from (19). (Inset) The (T, v) plane of the jamming phase diagram, with trajectories for glass transition and jamming.



way, $v_f(M)$ takes on a form identical to the Flory form (Eq. 2) for $T_g(M)$. Fitted to appropriate regimes of the $\rho_f(M)$ data, we find for $\xi = 7.5$, $v_b = 2.3$ and $l(v_b - v_{es}) = 2.6$; taking $l = \xi = 7.5$, then $v_{es} = 1.95$. For $\xi = 11.2$, the best fit gives $v_b = 2.5$, and $l(v_b - v_{es}) = 6$; taking $l = \xi = 11.2$ gives $v_{es} = 1.96$.

Finally, chains with larger loop sizes pack less densely than chains with small ξ . The analogy of $v_f(M)$ with $T_g(M)$ suggests that stiff polymers will have a higher T_g than those that are more flexible. For vinyl polymers with rigid side groups that restrict chain flexibility, T_g is higher for those with large side groups than for those whose side groups are small (20).

These observations do not prove that the polymer glass transition must be attributable to the jamming of chains, especially at finite temperatures and with realistic interactions. The role of temperature points to important distinctions between polymers and ball-chains: (i) The stiffness of a ball-chain is given solely by its construction, but a polymer becomes stiffer at lower T , and (ii) for the packing of macroscopic objects, thermal motion is irrelevant, and the generalization of temperature is an open question (21). Therefore, the purely geometric jamming of chains cannot be exactly analogous to the glass transition in polymers. Some aspects of temperature can perhaps be simulated by varying the tapping strength used to compact the packing (6, 22). Interaction can also be introduced, for instance, by making the chains slightly sticky with a thin coating of viscous oil. But even for hard particles and no explicit tem-

perature, the similarities between jammed ball-chains and glassy polymers are pronounced enough to suggest that the jamming idea captures much of the physics. Because it has long been a matter of debate whether glass transitions in polymers and in simple glass-formers are fundamentally similar, it is attractive to think that jamming, whether of grains or chains, may provide a unifying connection.

We have shown that long, floppy chains pack into a low-density structure whose rigidity is chiefly provided by the backbone. This can be understood as the jamming of semi-rigid loops that formed when the chains were compacted. By invoking an analogy between the specific packing volume and the glass transition temperature, we have shown that the packing of chains parallels the polymer glass transition in important respects. If these two phenomena are indeed closely connected, as our data suggest, it would be a beautiful illustration of how molecular geometry and symmetry, independent of the specific microscopic interactions, can influence the structure of condensed matter.

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Dirac Strings and Magnetic Monopoles in the Spin Ice $\text{Dy}_2\text{Ti}_2\text{O}_7$

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Sources of magnetic fields—magnetic monopoles—have so far proven elusive as elementary particles. Condensed-matter physicists have recently proposed several scenarios of emergent quasiparticles resembling monopoles. A particularly simple proposition pertains to spin ice on the highly frustrated pyrochlore lattice. The spin-ice state is argued to be well described by networks of aligned dipoles resembling solenoidal tubes—classical, and observable, versions of a Dirac string. Where these tubes end, the resulting defects look like magnetic monopoles. We demonstrated, by diffuse neutron scattering, the presence of such strings in the spin ice dysprosium titanate ($\text{Dy}_2\text{Ti}_2\text{O}_7$). This is achieved by applying a symmetry-breaking magnetic field with which we can manipulate the density and orientation of the strings. In turn, heat capacity is described by a gas of magnetic monopoles interacting via a magnetic Coulomb interaction.

Despite searching within the cosmic radiation, particle colliders, and lunar dust, free magnetic monopoles have not been observed (1, 2). This is particularly disappointing given that unification theories have predicted their existence. Dirac's original vision for monopoles involves a string singularity carrying magnetic flux, the ends of which act as north and south monopoles. We report the observation of analogous strings and magnetic monopoles in spin ice, magnetic compound $\text{Dy}_2\text{Ti}_2\text{O}_7$ with a pyrochlore lattice structure. This is a realization of magnetic fractionalization in three dimensions, a separation of north and south monopoles.

Dysprosium titanate contains magnetic ^{162}Dy ions in the highly frustrated pyrochlore lattice that have ferromagnetic exchange and dipolar interactions between the spins. The pyrochlore lattice is a three-dimensional (3D) structure built from corner-sharing tetrahedra (Fig. 1A). Spin ice is realized on this lattice when spins placed on the vertices are constrained to point radially into or out of the tetrahedra and are coupled ferromagnetically—or, as in the case of $\text{Dy}_2\text{Ti}_2\text{O}_7$, through dipolar coupling (3). This leads to the lowest-energy spin configurations obeying the “ice rules” of two spins pointing into, and two out of, each tetrahedron. This is equivalent to the physics of the proton

arrangement in ice, where two protons sit close to each oxygen and two far away—and indeed spin ice exhibits the Pauling ice entropy $S \approx (R/2) \ln(3/2)$ per spin (4, 5), reflecting a huge low-energy density of states in zero magnetic field.

Each spin can be thought of as a small dipole or solenoid channeling magnetic flux into and out of a tetrahedron. The ice rules are too weak to impose magnetic long-range order, but they do induce dipolar power-law correlations resulting in characteristic pinch-point features in neutron scattering (6–10).

A spin flip violates the ice rule in two tetrahedra, at a cost of ~ 2 K per tetrahedron in $\text{Dy}_2\text{Ti}_2\text{O}_7$. It was proposed that to a good approximation this can be viewed as the formation of a pair of monopoles of opposite sign in adjacent tetrahedra (11). These monopoles are deconfined (Fig. 1A); they can separate and move essentially independently. Thus, the equilibrium defect density is determined not by the cost of a spin flip but by the properties of the gas of interacting monopoles. In Fig. 1B, we compare the measured heat capacity to Debye-Hückel theory (12), which describes a gas of monopoles with Coulomb interactions. This theory is appropriate to low temperatures, where the monopoles are sparse, and it captures the heat capacity quantitatively. At higher temperatures, spin ice turns into a more conventional paramagnet and the monopole description breaks down (13). Together with a recent analysis of dynamic susceptibility (14), this lends strong support to the monopole picture of the low-temperature phase of spin ice.

Monopole deconfinement is reflected in the spin configurations: As the two monopoles of opposite sign separate, they leave a tensionless string of reversed spins connecting them. These strings of reversed flux between the monopoles can be viewed as a classical analog of a Dirac string. In the theory of Dirac (15), these are infinitely narrow, unobservable solenoidal tubes carrying magnetic flux density (**B**-field) emanating from the monopoles. Here, the strings are real and observable thanks to the preformed dipoles of the spins; strings can change length and shape

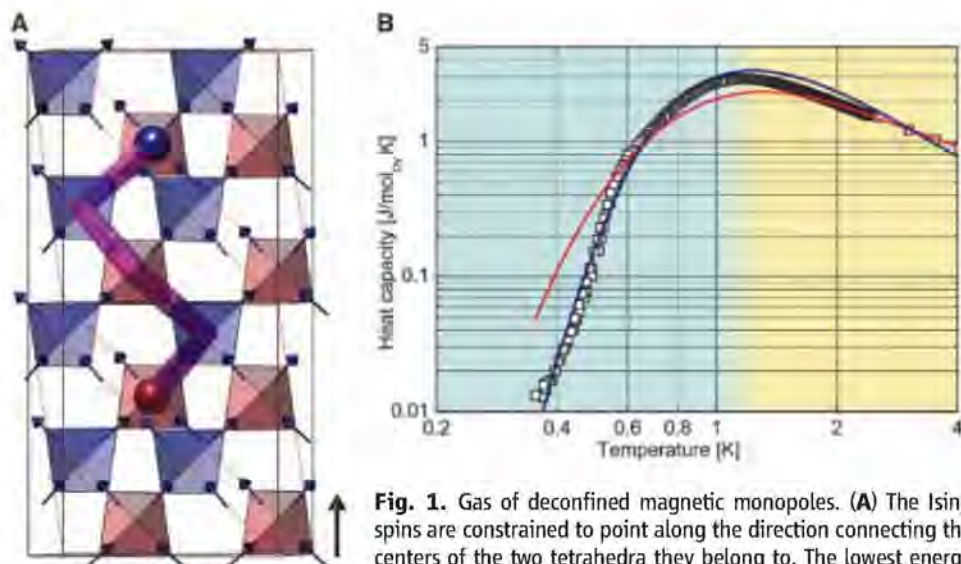


Fig. 1. Gas of deconfined magnetic monopoles. (A) The Ising spins are constrained to point along the direction connecting the centers of the two tetrahedra they belong to. The lowest energy for a tetrahedron is obtained for a two-in-two-out configuration, as illustrated. There are six such configurations with net ferromagnetic moments along one of the six equivalent $\langle 100 \rangle$ directions. The noncollinearity of the Ising axes is the source of the frustration in spin ice. In $\text{Dy}_2\text{Ti}_2\text{O}_7$ the “Ising” crystal field doublet is separated from other levels by more than 100 K. Applying a field, **B** || $[001]$, results in a preference for aligning the tetrahedral magnetization with the applied field direction (arrow). In the 3D pyrochlore lattice, Dirac strings of flipped spins terminate on tetrahedra where magnetic monopoles reside. (B) The measured heat capacity per mole of $\text{Dy}_2\text{Ti}_2\text{O}_7$ at zero field (open squares) is compared with a Debye-Hückel theory for the monopoles (blue line) and the best fit to a single-tetrahedron (Bethe lattice) approximation (red line). The ice-blue background indicates the spin-ice regime; the yellow background indicates the paramagnetic regime.

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at no cost in energy other than the magnetic Coulomb interaction between their endpoints.

Because the strings consist of magnetic dipoles, the method of choice for imaging them is magnetic neutron scattering. As a first step, using diffuse neutron-scattering techniques, we measured 3D correlation functions in $\text{Dy}_2\text{Ti}_2\text{O}_7$. Figure 2A shows the results with no applied magnetic field. One important property of the correlation functions in zero field is the existence of pinch points, a signature of the spin-ice state, which can be seen in the experiment as 3D singularities (fig. S1). For comparison with the neutron-scattering measurements, we performed a large- N (self-consistent mean-field) calculation (6, 16), supplemented with the relevant geometric factors for neutron-scattering experiments and the magnetic form factor of Dy (Fig. 2B). Good agreement between theory and experiment [see also (17)] demonstrates that the correlations do indeed follow the predicted dipolar form, even though direct observation of the pinch points is not possible because they are covered by Bragg peaks.

In the Dirac string picture, the spin-ice ground state satisfying the ice rules can be considered as a dense network of interwoven strings that are either closed (i.e., loops) or terminate at the surface of the sample. If the applied field is zero, the strings have no privileged orientation and they describe isotropic, intertwined 3D random walks of arbitrary length. Excitations correspond to monopoles at the end of Dirac strings (broken loops) in the bulk. The cost of lengthening such Dirac strings is solely against the weak attractive force between the monopoles at their ends (17). Indeed, as the strings fluctuate between different configurations consistent with a given distribution of monopoles, there is not even a unique way of tracing their paths.

However, there exists an elegant remedy: The application of a large magnetic field along one of the principal axes (here we choose the [001] direction) orients all spins. The resulting ground state is unique and free of monopoles; the ice rules are observed everywhere, and each tetrahedron is magnetized in the [001] direction. Upon lowering the field, sparse strings of flipped spins appear against the background of this fully mag-

netized ground state. In the absence of monopoles, such strings must span the length of the sample and terminate on the surface; otherwise, they can terminate on magnetic monopoles in the bulk (as explained above).

The presence or absence of the strings at a given temperature and field is determined by a balance between the energy cost of producing them and the gain in entropy due to their presence. As pointed out in (18, 19), each link in the string will involve a spin being reversed against the field (note that this still maintains the two-in-two-out ice rules along the string). Each spin flip costs a Zeeman energy of $(2/\sqrt{3})h$, where $h = gmB$ is the strength of the field applied along [001], g is the Landé g -factor, $m = 10\mu_B$ is the magnetic moment per Dy ion, and B is the applied field. As there are two possibilities to choose for the continuation of the string, there is an associated entropy per link of $s = k_B \ln(2)$, where k_B is the Boltzmann constant. The free energy per link, as the string becomes large, is

$$f = u - Ts = \frac{2}{\sqrt{3}}h - k_B T \ln(2) \quad (1)$$

where u is the Zeeman energy and T is absolute temperature. For fields above $h_K = [k_B T \ln(2)] / (2/\sqrt{3})$, the number of strings goes to zero as the free energy of formation is macroscopic and positive. However, at the Kasteleyn field h_K , a transition occurs where strings spontaneously form as the free energy becomes favorable and the entropy of string formation wins. This transition is a 3D example of a Kasteleyn transition (18), a highly unusual topological phase transition.

Thus, by measuring close to this transition, we can dial up a regime where the strings are sparse and oriented against the field direction. This allows us to check, qualitatively and quantitatively, the properties of these strings; in particular, we find that they lead to a qualitative signature in the neutron scattering, which can be manipulated by tilting the field. In the following, we first locate the Kasteleyn transition and hence h_K , and then discuss the neutron-scattering data in detail by comparing them to a theoretical

model. Evidence of this transition can be seen in the magnetization as a function of temperature and field along [001] (Fig. 3, A and B). Whereas the magnetization in response to field changes at constant temperature only equilibrates above 0.6 K, spin ice can remain closer to its thermodynamic equilibrium behavior when cooled in zero magnetic field. Above this temperature, the system shows a transition to saturation at a field $h_S(T)$ consistent with that expected for the 3D Kasteleyn transition. Indeed, in the ergodic region of the phase diagram the saturation field h_S coincides with the Kasteleyn field h_K , where a kink in the magnetization appears as it reaches its saturation value (18). However, below ~ 0.6 K, equilibration times become so long that the system starts freezing (18), and the measured magnetization is no longer an equilibrium property. Another signature of this freezing (Fig. 3B) is that the saturation field h_S (dotted white line) becomes temperature-independent.

Here, we restrict ourselves to equilibrium phenomena: All the neutron measurements in this study were undertaken above 0.6 K. These experimental temperatures are high enough, relative to the creation energy of monopoles, that the assumption of perfect compliance to the ice rules is no longer valid and the transition is rounded: The (low) thermal density of monopoles leads to strings of finite length.

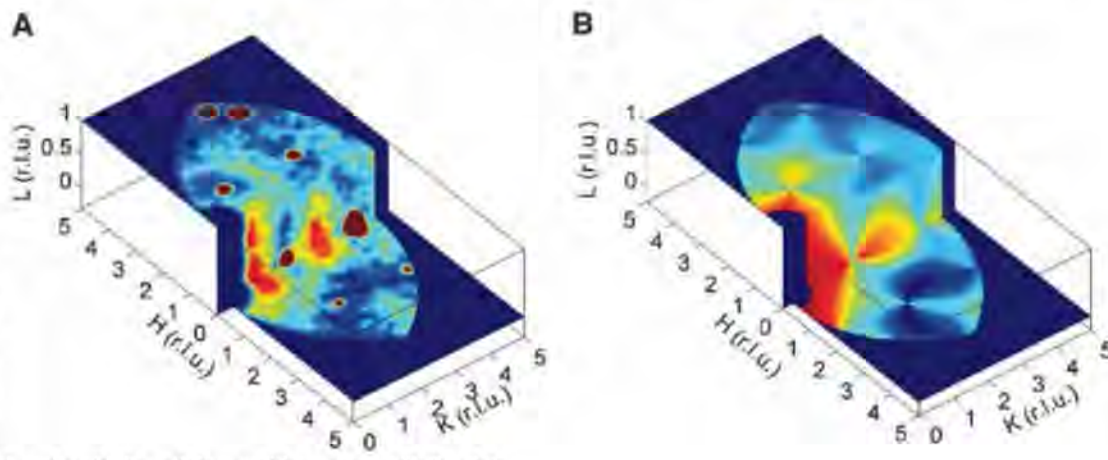
Figure 3C shows reciprocal space slices at a field near saturation of $h = 5/7 h_S$. Instead of the two lobes coming down to a pinch point in zero field, cone-like scattering emanates from what was the position of the pinch point. As the field is decreased, the diffuse scattering smoothly deforms back to the zero-field form.

As described above, the strings execute a random walk; when their density is small, interactions between them can be neglected to a first approximation, so that the spin correlations are those of a diffusion process with the z coordinate assuming the role usually played by time:

$$C(x, y, z) \approx \frac{1}{z} \exp\left(\gamma \frac{x^2 + y^2}{z}\right) \quad (2)$$

where γ is a geometric constant (13).

Fig. 2. 3D rendering of the dipolar correlations in reciprocal space (hkl) of spin ice at 0.7 K. (A) Neutron diffraction data taken at 0.7 K and 0.0 T on the flat-cone diffractometer E2, at HZB, with the diffuse peaks at (030) and minima in $(\frac{3}{2}, \frac{5}{2}, 0)$ positions (r.l.u., reciprocal lattice units). Bragg peaks (red spots) lie on top of the pinch points. A secondary Bragg peak is from a smaller crystallite. (B) Pinch points are found in the correlation functions and these are 3D in nature, with the diffuse scattering constricting at the reciprocal lattice point (020) and its equivalents. Diffuse peak and scattering minima positions agree with the data.



To capture lattice effects, we simulate random walks on the pyrochlore lattice. The correlations we find are essentially unchanged if we include interactions in the form of hard-core exclusion (13). Because there is a finite thermal population of monopoles and defects in the material, we expect the strings to be finite in length. For the field of $h = 5/7 h_S$ and 0.7 K, a string length on the order of 50 sites is required for agreement with the data. Indeed, for this temperature in zero field, the density of monopoles in numerical simulations is found to be very low—well below 1% of all tetrahedra—in keeping with a large string length.

The scattering from a large ensemble of such hard-core walks has been calculated including all the geometrical factors for the neutron-scattering cross section. As can be seen from the side-by-side comparison of the data and modeling, the string configurations account very well for the data and reproduce the cone of scattering observed.

We repeated the experiment with an effective field tilted $\sim 10^\circ$ toward the [011] direction to induce a net tilt in the meandering of the strings. The cone of diffuse scattering collapses into sheets of scattering at an angle of 45° , matching the opening angle of the original cone. This sharp sheet in reciprocal space widens with decreasing field (Fig. 4C). Within the random-walk model, tilting the applied magnetic field changes the relative probabilities of each step (thus generat-

ing a biased random walk) as inequivalent spin flips incur different energy costs. The field and temperature-dependent Boltzmann factor for the ratio of probabilities of stepping from tetrahedral site 1 to site 3, versus site 1 to site 4, is

$$\frac{p(1 \rightarrow 3)}{p(1 \rightarrow 4)} = \exp \left[\frac{2m|h|(\cos \theta_1 - \cos \theta_2)}{k_B T} \right] \quad (3)$$

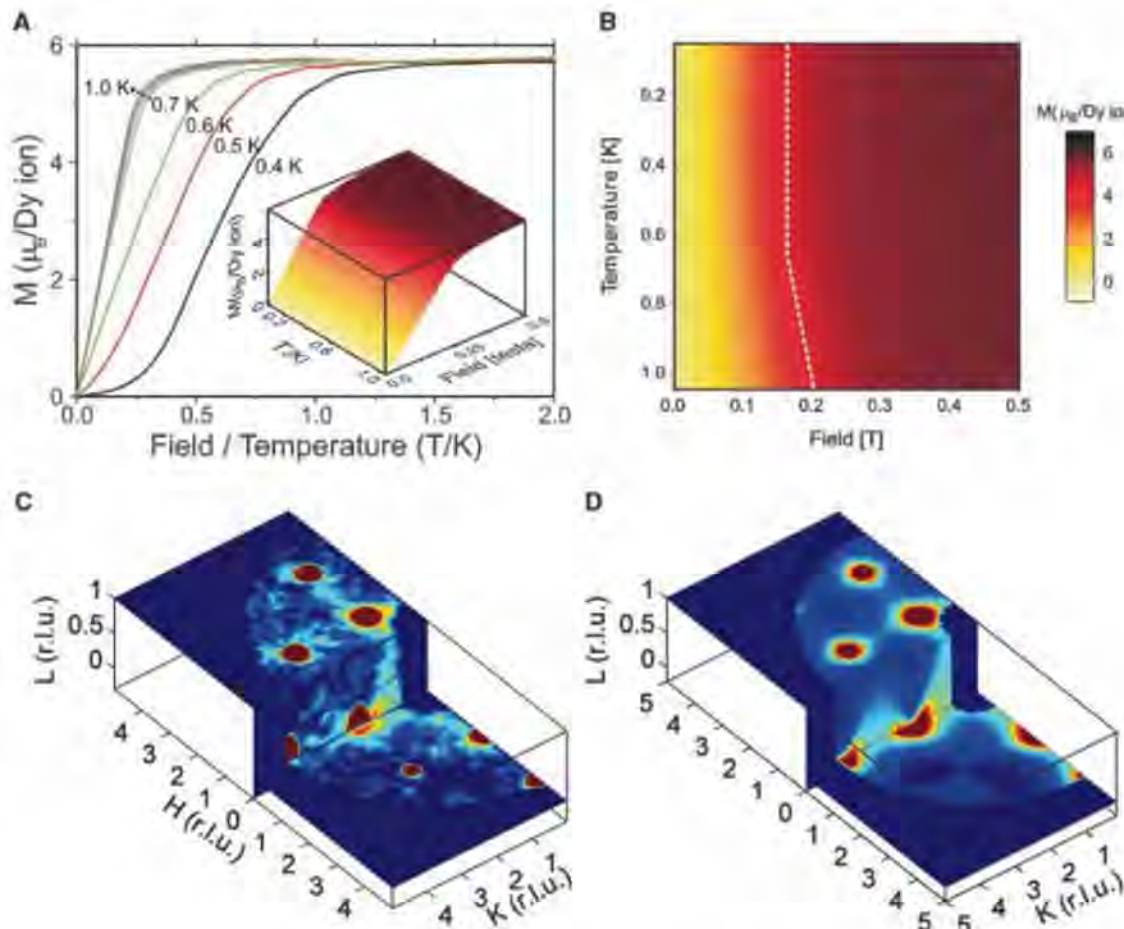
where θ_1 and θ_2 are the angles between the magnetic moments on the two final sites and the magnetic field $\mathbf{h}$. Because of the ferromagnetism induced in spin ice, demagnetization effects must be carefully accounted for in the modeling. A bias of 0.8:0.2 at $4/7 h_S$ and 0.64:0.36 at $2/7 h_S$ is anticipated from Eq. 3. Using these weighting factors and modeling the new ensemble of Dirac strings, the tilts and widths of the scattering are well reproduced.

There still remains the issue of interstring correlations. Comparison with the hard-core string model (Fig. 4, C and D) shows that the simple random-walk approach does not capture the intensity distribution so well. Figure 4D shows a cut through the walls of scattering in the $(h, 2\eta + l, l)$ plane, where η is an integer. The intensity within the sheets is indicative of correlations between strings. This may indicate short-range ordering of the strings, and the increased intensities around $(2, 2/3, 2/3)$ would suggest a local hexagonal patterning. Further

calculations are needed, including interactions between strings, to clarify this in detail.

Our study of the spin-ice state in zero field and under an applied magnetic field along [001] lends support to the strongly correlated and degenerate nature of the ground state, as well as the resulting long-range dipolar correlations. The low-energy excitations of such a complex ground state are remarkable in their simplicity and can largely be accounted for by weakly interacting point-like quasiparticles (the magnetic monopoles) connected by extended objects (the Dirac strings of reversed spins). In zero field, a description based on a gas of monopoles accounts for the measured low-temperature specific heat. Under fields applied along [001], the picture is that of Dirac strings of reversed spins meandering along the direction of the applied field and terminating on monopoles. This picture accounts very well for the spin correlations observed through neutron scattering. The behavior of the magnetization as a function of temperature and field near saturation, where all the strings are expelled, is an example of a (thermally rounded) 3D Kasteleyn transition. This description is rather robust and gives a simple picture of the spin configuration under tilted fields in terms of biases in the string direction. Our main result consists of the experimental identification of these string-like spin excitations in a gas of magnetic monopoles. These constitute hardy and practical

Fig. 3. Magnetization and diffuse neutron scattering with field applied along [001]. (A) Magnetization plotted versus [001] field over temperature for fixed temperatures of 0.4, 0.5, 0.6, 0.7, 0.85, 0.9, and 1.0 K, showing a clear departure from h/T scaling at 0.6 K and below. Inset: Surface of magnetization as a function of temperature and field as constructed from more than 70 field and temperature sweeps. (B) Contour plot of the magnetization as a function of field and temperature. The dotted white line shows h_S , which is seen to freeze below 0.6 K. (C) 3D representation of the single-crystal neutron diffraction data from E2, HZB, at $5/7 h_S$ and 0.7 K, showing a cone of scattering coming from the (020) Bragg peak. (D) Calculation of diffuse scattering characteristic of the weakly biased random-walk correlations with bias of 0.53:0.47 and $\mathbf{B}_{\text{int}} \parallel [001]$.



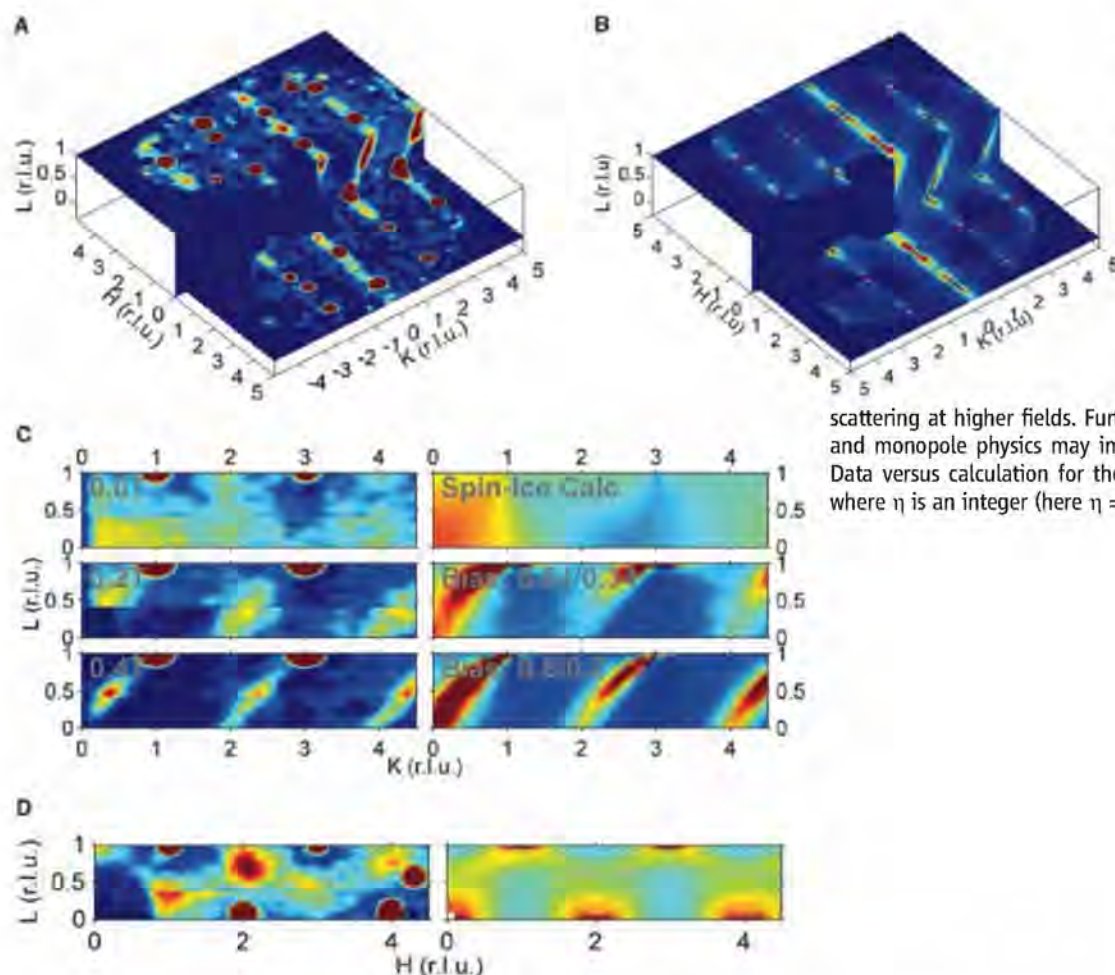


Fig. 4. Biased random walks in a tilted field. **(A)** Neutron diffraction data from E2, HZB, showing the $(hk0)$, $(hk1)$, and $(0kl)$ planes taken at 0.7 K and a field of $4/7h_s$. The red spots are Bragg peaks; the peaks at $(2.7, -1.8, 0)$, $(1.3, -2.3, 1)$, $(3.8, -0.9, 1)$, and $(3.5, 2.5, 1)$ are from a smaller second crystallite. **(B)** Random-walk string model with biasing 0.8:0.2. **(C)** Field dependence of the diffuse scattering and calculations in the $(1, k, l)$ plane. Spin-ice scattering collapses into walls of scattering at higher fields. Further understanding of the Dirac string and monopole physics may improve our modeling of the data. **(D)** Data versus calculation for the $(h, 2\eta + l, l)$ diffuse wall of scattering, where η is an integer (here $\eta = 0$).

building blocks for the understanding of the low-energy behavior of spin ice. Perhaps the most intriguing open issue is the precise connection between these building blocks and the low-temperature freezing observed in the spin-ice compounds (14, 20).

Our work constitutes direct evidence of Dirac strings. It provides compelling evidence for the dissociation of north and south poles—the splitting of the dipole—and the identification of spin ice as the first fractionalized magnet in three dimensions. The emergence of such striking states is profoundly important in physics, both as a manifestation of new and singular properties of matter and as a route to potential technologies. Examples of fractionalization are extremely rare and almost exclusively pertain to one and two dimensions, and so the 3D pyrochlore lattice offers a promising direction for future exploration in both magnets and exotic metals.

Our findings are of relevance not only from a fundamental physics aspect—we have evidenced a set of quasiparticles that have no elementary cousins—but also because they imply a new type of degree of freedom in magnetism, namely an object with both local (point-like monopole) and extended (tensionless Dirac string) properties. $\text{Dy}_2\text{Ti}_2\text{O}_7$ is an exceptionally clean material, and with the full array of powerful experimental techniques and pulsed fields, equilibrium and non-

equilibrium properties can be comprehensively addressed, although this will present a substantial statistical physics and dynamical systems challenge. The results of such studies may shed light on other systems where string-like objects can appear—for instance, in the study of polymers or nanoclusters—but where freezing of solvents and inhomogeneities can restrict access to all the physics. Spin ice promises to open up new and complementary insights on both the emergence of fractionalized states and the physics of ensembles of strings in and out of equilibrium.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1178868/DC1
Materials and Methods
Figs. S1 to S13
Table S1
References

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Magnetic Coulomb Phase in the Spin Ice $\text{Ho}_2\text{Ti}_2\text{O}_7$

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Spin-ice materials are magnetic substances in which the spin directions map onto hydrogen positions in water ice. Their low-temperature magnetic state has been predicted to be a phase that obeys a Gauss' law and supports magnetic monopole excitations: in short, a Coulomb phase. We used polarized neutron scattering to show that the spin-ice material $\text{Ho}_2\text{Ti}_2\text{O}_7$ exhibits an almost perfect Coulomb phase. Our result proves the existence of such phases in magnetic materials and strongly supports the magnetic monopole theory of spin ice.

The spin ice family (1–3) is part of a larger class of magnetic materials that at low temperatures enter a thermodynamic state known as a cooperative paramagnet (4, 5). The spins are correlated, but competing interactions prevent long range order, so that the system fluctuates, exploring its many degenerate ground states. Typically, there is some local rule that can be used to construct these ground states. For example, in a spin ice ground states are obtained by ensuring that a “two spins in, two spins out” configuration is satisfied on every tetrahedron (Fig. 1A). Similar rules, generically termed “ice rules,” control hydrogen atom positions in water ice (6), dimer configurations in spin liquids (7), and spin configurations in Heisenberg pyrochlore antiferromagnets (8). In theory, all these systems are effective Coulomb phases (9, 10) because the ice rule variables can be mapped to a nondivergent field, and excitations that break the constraint create effective monopoles (11, 12) in that field. The system obeys a Gauss' law, which relates divergences in a field to a pole density.

Theoretical work (10, 13) has recognized that the fundamental difference between a conventional paramagnet and a magnetic Coulomb phase is in the form of the spin correlation function at a large distance [see also (14) for the analogous case of paraelectrics]. In the former, the spin correlation function decays like a screened Coulomb interaction; $\frac{1}{r} \exp^{-\kappa r}$, where κ is the inverse correlation length and r is the distance, whereas in the latter it is predicted to decay like a dipolar interaction $\sim \nabla_r \nabla_r \cdot \frac{1}{r} = \frac{1}{r^3}$. The dipolar form of the spin correlation function represents the Gauss's law (without poles) of the Coulomb phase and in principle affords an unambig-

uous experimental signature for it. Thus, the pseudo-dipolar correlations in direct (“real”) space Fourier transform into a set of pinch-point singularities, resembling bow-ties, in the reciprocal space probed by a scattering experiment. Although pinch points have been predicted for various magnetic systems, they have only been clearly observed in the field-induced kagome ice phase of spin ice (15, 16). They appear to be absent in all candidate magnetic Coulomb phases (17–19), including the zero-field spin ice state (15, 20–23).

Previous unpolarized neutron scattering on spin ices, such as $\text{Ho}_2\text{Ti}_2\text{O}_7$ (1, 3), has established that the dipolar spin ice model, in which the rare-earth ions are coupled by the dipole-dipole interaction and competing superexchange, gives an accurate description of bulk and microscopic properties (20, 23). The underlying reason for the persistence of spin ice behavior in this more complex model is that the dipolar Hamiltonian

has been shown to have practically identical groundstates to the near-neighbor (ice rules) model (10, 24), a feature known as projective equivalence. The two differ by small corrections that are expected to vanish as r^{-5} . It is therefore strongly expected that the spin-spin correlations of $\text{Ho}_2\text{Ti}_2\text{O}_7$ should exhibit a pseudo-dipolar form.

The combination of both dipolar correlations and interactions is essential to the proposal that ice rule defects in $\text{Ho}_2\text{Ti}_2\text{O}_7$ and $\text{Dy}_2\text{Ti}_2\text{O}_7$ are genuine magnetic monopoles (11). A Gauss' law is obeyed by the magnetization $M(r)$ and the magnetic H -field. Monopolar sources and sinks in M and H correspond to thermally excited spin flips (Fig. 1B) (11, 25). Although the Gauss' law is a consequence of the dipolar spin correlations, it is the physical dipolar interactions that cause the relevant fields to be the electrodynamic quantities M and H . The dipolar interactions are not in doubt, but the failure to resolve a pinch point in the zero-field spin ice state (15, 20–23) raises questions about the reality of the dipolar correlations and hence the theories built upon them: the accuracy of projective equivalence, the postulated magnetic Coulomb phase, and the existence of magnetic monopoles.

Neutron scattering estimates the scattering function $S^{\alpha\beta}(\mathbf{Q})$ in reciprocal space (here $\alpha, \beta = x, y, z$), which is the required Fourier transform of the thermally averaged two-spin correlation function. Our polarized neutron experiments were configured to measure two independent components of the tensor $S^{\alpha\beta}(\mathbf{Q})$ that we label as spin flip (SF) and non-spin flip (NSF). Here, z is vertical and x (y) is defined to be parallel (perpendicular) to the scattering vector $\mathbf{Q}$ in the horizontal scattering plane. With vertical (or z)

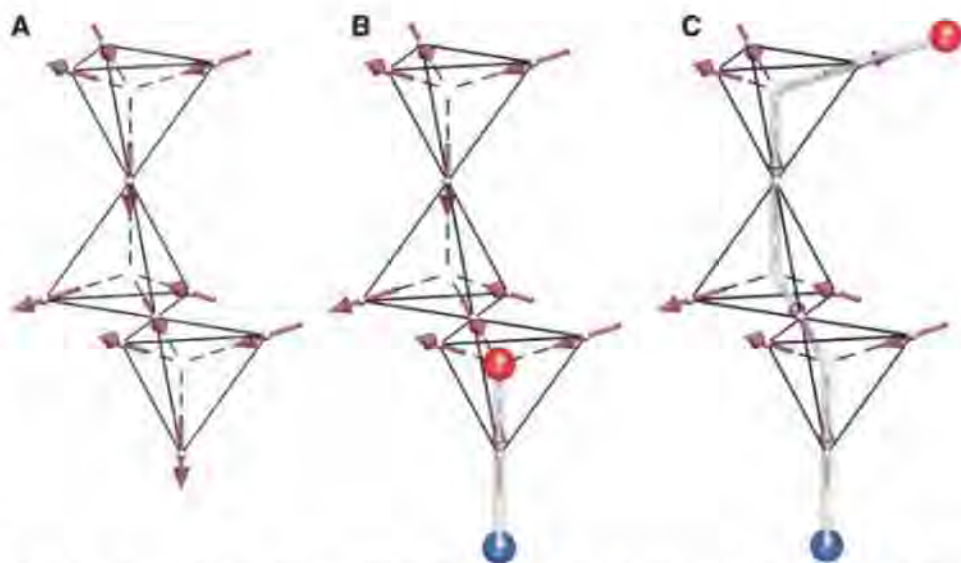


Fig. 1. Fragment of the spin ice structure. (A) The spin ice state in which each tetrahedron has the “two spins in, two spins out” configuration. (B) A single spin flip produces defects on two neighboring tetrahedra. (C) The defects can move apart. They interact like oppositely charged magnetic monopoles connected by a trail of flipped spins (a Dirac string). The pink arrows indicate spins, the blue spheres indicate monopoles, and the red spheres indicate antimonopoles.

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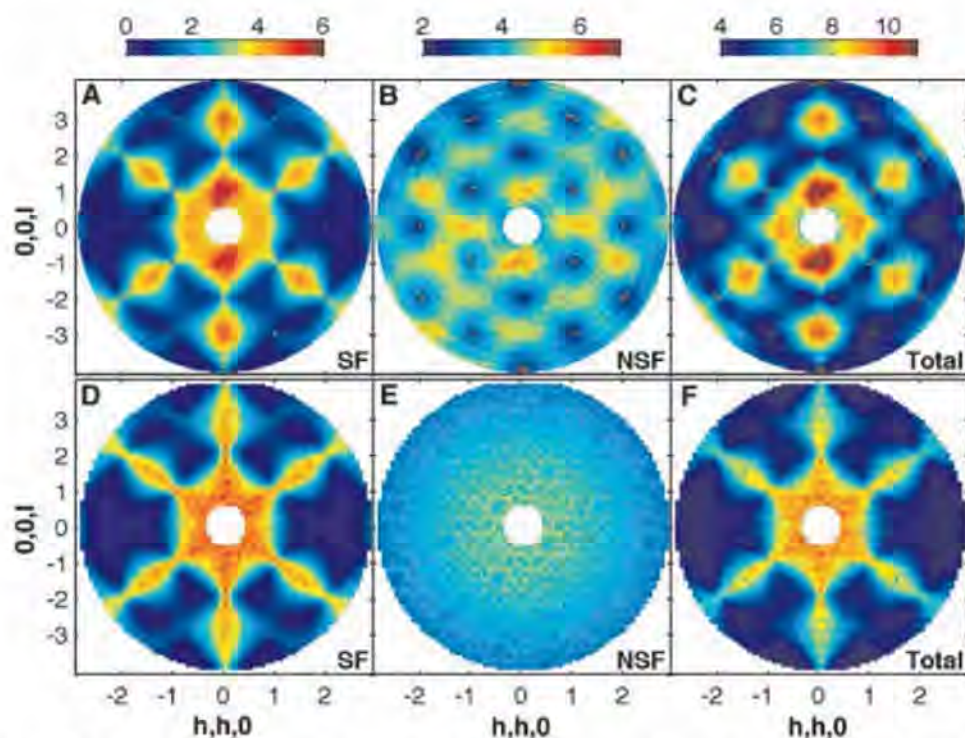


Fig. 2. Diffuse scattering maps from spin ice, $\text{Ho}_2\text{Ti}_2\text{O}_7$. Experiment [(A) to (C)] versus theory [(D) to (F)]. (A) Experimental SF scattering at $T = 1.7$ K with pinch points at $(0, 0, 2)$, $(1, 1, 1)$, $(2, 2, 2)$, and so on. (B) The NSF scattering. (C) The sum, as would be observed in an unpolarized experiment (20, 22). (D) The SF scattering obtained from Monte Carlo simulations of the near-neighbor model, scaled to match the experimental data. (E) The calculated NSF scattering. (F) The total scattering of the near-neighbor spin ice model.

incident neutron polarization, the SF and NSF cross sections yield information on $S^{yy}(\mathbf{Q})$ and $S^{\pm}(\mathbf{Q})$, respectively. We used a single crystal of $\text{Ho}_2\text{Ti}_2\text{O}_7$ to map diffuse scattering in the h, h, l plane. Previous unpolarized experiments (20, 22) have measured the sum of the SF and NSF scattering, but in this orientation only the SF scattering would be expected to contain pinch points (26).

Our results (Fig. 2A) show that at temperature ($T = 1.7$ K) there are pinch points in the SF cross section at the Brillouin zone centers $(0, 0, 2)$, $(1, 1, 1)$, and $(2, 2, 2)$ (Fig. 2A) but not in the NSF channel (Fig. 2B). The total scattering (SF + NSF) reveals the pinch points only very weakly (Fig. 2C) because the NSF component dominates near the zone center. This is explicitly illustrated with cuts across the zone center showing that the strong peak at the pinch point in the SF channel is only weakly visible in the total (Fig. 3B). The total scattering (Figs. 2C and 3B) can be compared with the previous observations and calculations (20, 22), in which no pinch points were detected. The use of polarized neutrons extracts the pinch-point scattering from the total scattering, and the previous difficulty in resolving the pinch point is clearly explained.

The projective equivalence of the dipolar and near-neighbor spin ice models (10) suggests that above a temperature scale set by the r^{-5} corrections, the scattering from $\text{Ho}_2\text{Ti}_2\text{O}_7$ should

become equivalent to that of the near-neighbor model. $T = 1.7$ K should be sufficient to test this prediction because it is close to the temperature of the peak in the electronic heat capacity that arises from the spin ice correlations [1.9 K (20)]. In our simulations of the near-neighbor spin ice model (Fig. 2, D to F), the experimental SF scattering (Fig. 2A) appears to be very well described by the near-neighbor model, whereas the NSF scattering is not reproduced by the theory. However, we have discovered that $S(\mathbf{Q})^{\text{experiment}}/S(\mathbf{Q})^{\text{theory}}$ is approximately the same function $f(\mathbf{Q})$ for both channels. Thus, because the theoretical NSF scattering function is approximately constant, we find $f(\mathbf{Q}) \approx S(\mathbf{Q})^{\text{experiment}}_{\text{NSF}}$. This function may be described as reaching a maximum at the zone boundary and a finite minimum in the zone center. Using the above estimate of $f(\mathbf{Q})$, the comparison of the quantity $S(\mathbf{Q})^{\text{experiment}}_{\text{SF}}/f(\mathbf{Q})$ with $S(\mathbf{Q})^{\text{theory}}_{\text{SF}}$ is considerably more successful. Differences are less than 5% throughout most of the scattering map (26).

Cuts through the pinch point at $(0, 0, 2)$ at 1.7 K (Fig. 3, A and B) show that it has the form of a low sharp saddle in the intensity. In order to better resolve the line shape of the pinch point, we performed an analogous polarized neutron experiment on a higher-resolution spectrometer. To compare with theory, we used an approximation to an analytic expression (13, 27).

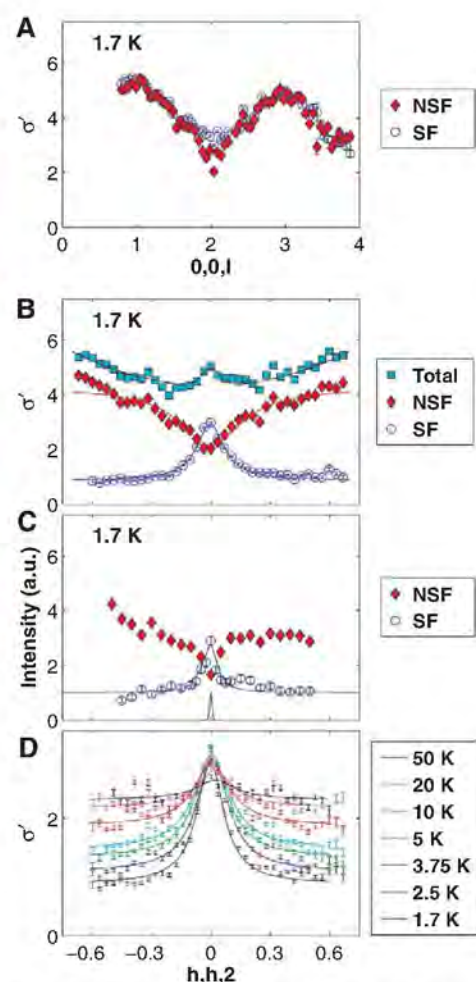


Fig. 3. Line shape of the pinch point. (A) Radial scan on D7 through the pinch point at $(0, 0, 2)$ [σ' is the neutron scattering cross section; see (26) for its precise definition]. (B) The corresponding transverse scan. The lines are Lorentzian fits. (C) Higher-resolution data, in which the line is a resolution-corrected fit to the pinch point form Eq. 1 (the resolution width of the spectrometer is indicated as the central Gaussian). (D) SF scattering at increasing temperatures (the lines are Lorentzians on a background proportional to the Ho^{3+} form factor).

In the vicinity of the $(0, 0, 2)$ pinch point, this becomes

$$S^{yy}(q_h, q_k, q_l) \propto \frac{q_{l-2}^2 + \xi_{\text{ice}}^{-2}}{q_{l-2}^2 + q_h^2 + q_k^2 + \xi_{\text{ice}}^{-2}} \quad (1)$$

Here, ξ_{ice} is a correlation length for the ice rules that removes the singularity at the pinch point (27). The high-resolution data of Fig. 3C can be described by this form, with a correlation length $\xi_{\text{ice}} \approx 182 \pm 65$ Å, representing a correlation volume of about 14,000 spin tetrahedra. The correlation length has a temperature variation that is consistent with an essential singularity $\sim \exp(B/T)$, with $B = 1.7 \pm 0.1$ K (Fig. 4C).

The scattering in the NSF channel is concentrated around Brillouin zone boundaries, as

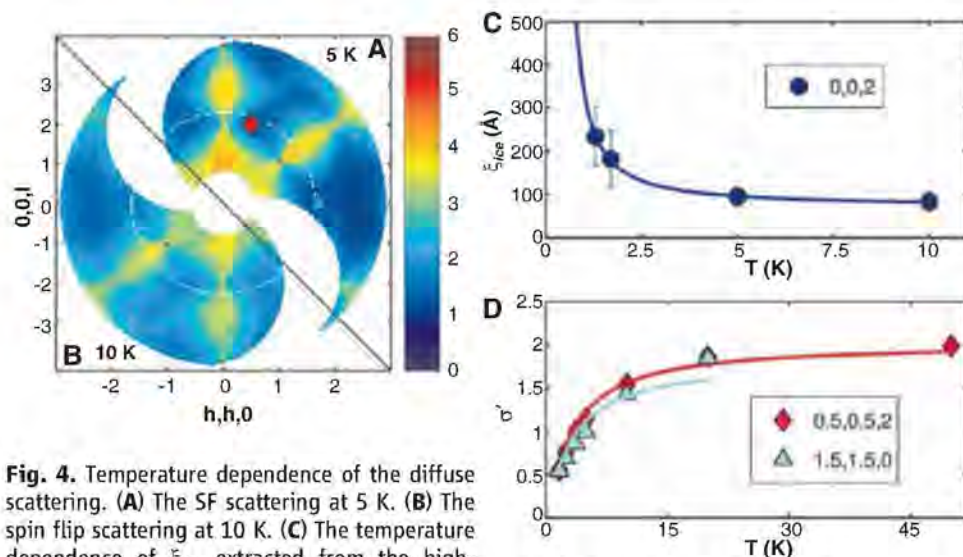


Fig. 4. Temperature dependence of the diffuse scattering. **(A)** The SF scattering at 5 K. **(B)** The spin flip scattering at 10 K. **(C)** The temperature dependence of ξ_{ice} extracted from the high-resolution data fitted to an exponential divergence in T^{-1} . **(D)** Temperature dependence of the cross section σ' at (0.5, 0.5, 2) (the pinch-point background in Fig. 3D) and (1.5, 1.5, 0) [indicated by corresponding symbols in (A)]. The lines are fits to Eq. 2 (there is a fitted scale factor).

previously observed in unpolarized cross sections for both $\text{Ho}_2\text{Ti}_2\text{O}_7$ (22) and $\text{Dy}_2\text{Ti}_2\text{O}_7$ (21, 23). The NSF scattering shows a pronounced symmetric minimum at each Brillouin zone center, which is roughly as sharp as the maximum in the SF scattering (Fig. 3C). The sharp but finite minimum indicates an effect that tends to suppress long-range spin correlations but fails to do so completely: The pinch point and associated dipolar correlations remain. The distinct structure of the NSF scattering (which persists to temperatures as high as 10 K) suggests a simple and generic correction to the near-neighbor model emerging from the dipolar interaction. The low-temperature evolution of the zone boundary scattering suggests that it is linked to corrections that become more important at low temperatures (22).

The general effect of increasing temperature on the SF scattering pattern (Fig. 4A) shows that empty areas of $S^{1/2}$ (for example, near 1.5, 1.5, 0) are increasingly filled in as the temperature rises, with a thermal contribution that is independent of wave vector (apart from the Ho^{3+} form factor), indicating uncorrelated point defects. We identify these as monopoles that remain strongly bound as dipole pairs (Fig. 1B). This behavior is also illustrated in Fig. 3D, in which, with increasing temperature, there is a marked decrease in peak intensity and an increase in the background on which the peak stands. As shown in Fig. 4D, this contribution can be generally fitted by the form

$$I(T) \propto \frac{\exp(-2J/T) + \exp(-8J/T)}{1 + \exp(-2J/T) + \exp(-8J/T)} \quad (2)$$

where $I(T)$ is the intensity and $J = J_{\text{eff}} = 1.8$ K, which is the effective, near-neighbor exchange

that is appropriate for $\text{Ho}_2\text{Ti}_2\text{O}_7$ (3). The two terms in the numerator are the cost of creating “singly charged” (monopole) or “doubly charged” thermal defects in the ice rules, respectively.

Two monopoles created by a spin flip can diffuse apart, leaving a path of successive head-to-tail spins, which is known as a Dirac string (Fig. 1C) by analogy with Dirac’s theory of magnetic monopoles. In spin ice, the strings carry local magnetization. If strings exist with lengths up to $\sim \xi_{\text{ice}}$, then this should be manifested as an approximately Lorentzian scattering with width ξ_{ice}^{-1} . Hence, we can attribute the broadening of the pinch point to the existence of unbound defects connected by Dirac strings with lengths up to ξ_{ice} (11, 25). At high temperatures, the proliferation of bound defects will both disrupt existing strings and reduce the mean free path for diffusing monopoles, reducing the maximum length in the Dirac string network. As the temperature is reduced, the thermal defect population decreases, and ξ_{ice} diverges as approximately $\exp(B/T)$ (Fig. 4C), with the observed value of B close to the effective exchange $J_{\text{eff}} = 1.8$ K (3). Such a temperature variation of ξ_{ice} is the same as that of the correlation length of the one-dimensional Ising ferromagnet, which is indeed the maximum length of a ferromagnetic string in that system.

Investigation of the spin ice $\text{Ho}_2\text{Ti}_2\text{O}_7$ by use of polarized neutron scattering has established the validity of projective equivalence (24) and quantified the corrections to it. We have established that $\text{Ho}_2\text{Ti}_2\text{O}_7$ exhibits an almost ideal magnetic Coulomb phase, the quasi-particle vacuum for magnetic monopoles (11, 25). We have shown that bound monopole pairs dominate at finite temperature, but that unbound pairs become relatively more important at low temperatures. The length of the longest Dirac

strings has been estimated to rise to macroscopic scales as the temperature passes below 1 K.

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Tyrannosaurid Skeletal Design First Evolved at Small Body Size

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Nearly all of the large-bodied predators (>2.5 tons) on northern continents during the Late Cretaceous were tyrannosaurid dinosaurs. We show that their most conspicuous functional specializations—a proportionately large skull, incisiform premaxillary teeth, expanded jaw-closing musculature, diminutive forelimbs, and hindlimbs with cursorial proportions—were present in a new, small-bodied, basal tyrannosauroid from Lower Cretaceous rocks in northeastern China. These specializations, which were later scaled up in Late Cretaceous tyrannosaurids with body masses approaching 100 times greater, drove the most dominant radiation of macropredators of the Mesozoic.

Tyrannosaurus rex is the best-known of several tyrannosaurid species (1), which were dominant in their role as multi-ton predators on northern continents during the final 25 million years of the Mesozoic (2–4). Hallmark tyrannosaurid adaptations include a relatively large skull with enhanced jaw-closing musculature, enlarged olfactory bulbs, a relatively miniaturized forelimb with only two functional digits, and a pinched pedal structure common to cursors—features pre-

sumed to have evolved for hypercarnivory (strict carnivory) at large body size. Tyrannosaurids have been viewed as heterochronic “peramorphs” (5), the largest of which grew beyond the size and form of smaller-bodied ancestors (6) via developmental acceleration (7).

In the past decade, fossils pertaining to earlier and more primitive tyrannosauroid species have been discovered in rocks of Middle Jurassic to Early Cretaceous age in North America, Europe,

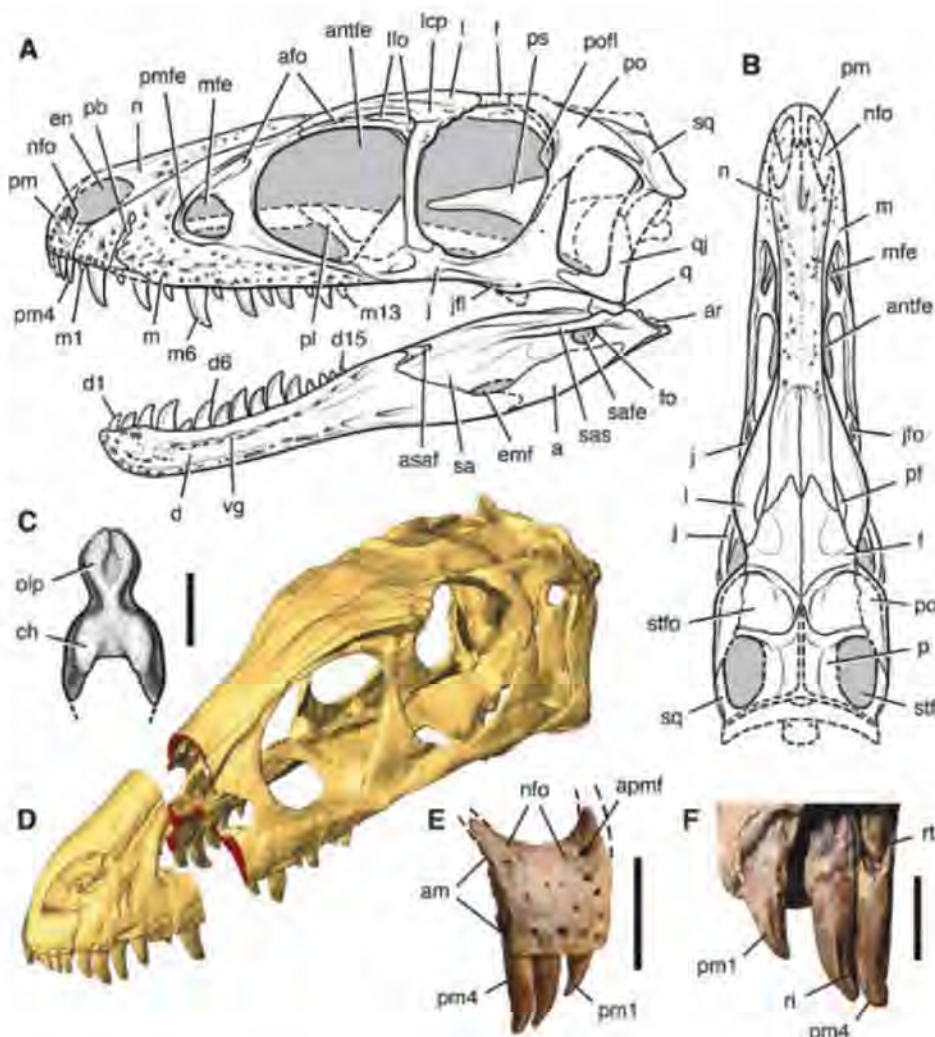
and China, including *Guanlong* (8), *Dilong* (5), and *Xiongguanlong* (9) and the more fragmentary *Eotyrannus* (10), *Stokesosaurus* (11), *Aviatyrannis* (12), and *Proceratosaurus* (13). With body lengths of only 2 to 5 m, these finds confirmed that tyrannosaurids evolved from small-bodied, long-armed “tyrannoraptors” (1, 4, 10). Evidence linking these precursors to their oversized descendants has been limited to a relatively small set of derived features in the skull and skeleton (5, 8, 9). On the other hand, it has been thought that the much greater range of truly tyrannosaurian characters, particularly those in the skull and limbs, evolved only with body size increase in Late Cretaceous tyrannosaurids (14).

We describe here a new small-bodied theropod, *Raptorex kriegsteini* nov. gen. nov.

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Fig. 1. Skull, endocast, and premaxillary teeth of the Early Cretaceous tyrannosauroid *R. kriegsteini*. The skull reconstruction in (A) lateral and (B) dorsal views is shown. (C) Partial endocast in dorsal view showing the enlarged olfactory peduncles and swollen cerebral hemispheres. (D) Skull model composed of cast bones as seen in a computed tomography scan showing a cross section (red) of the snout at mid-length. (E) Right premaxilla in lateral view. (F) Right premaxillary crowns in posteromedial view. Abbreviations: a, angular; afo, accessory fossa; am, articular surface for the maxilla; antfe, antorbital fenestra; apmf, anterior premaxillary foramen; ar, articular; asaf, anterior surangular foramen; ch, cerebral hemisphere; d, dentary; d1, d6, and d15, dentary tooth 1, 6, and 15; emf, external mandibular fenestra; en, external naris; f, frontal; fo, foramen; j, jugal; jfl, jugal flange; jfo, jugal fossa; lcp, lacrimal cornual process; l, lacrimal; lfo, lacrimal fossa; m, maxilla; m1, m6, and m13, maxillary tooth 1, 6, and 13; mfe, maxillary fenestra; n, nasal; nfo, narial fossa; olp, olfactory peduncle; p, parietal; pb, pathologic bone; pf, prefrontal; pl, palatine; pm, premaxilla; pm1 and pm4, premaxillary tooth 1 and 4; pmfe, premaxillary fenestra; po, postorbital; pofl, postorbital flange; ps, parasphenoid; q, quadrate; qj, quadratojugal; ri, ridge; rt, replacement tooth; sa, surangular; safe, surangular fenestra; sas, surangular shelf; sq, squamosal; stf, supratemporal fenestra; stfo, supratemporal fossa; vg, vascular groove. Scale bars, 3 cm in (C), 2 cm in (E), and 1 cm in (F).



sp. (15), that exhibits all major tyrannosaurid functional specializations in the skull and skeleton (Figs. 1 and 2), including a diminutive

forelimb (Fig. 3). Discovered in the Lujiatun Beds [Hauterivian-Barremian stages, ~130 million years ago (Ma)] of the Lower Cretaceous

Jehol Group in northeast China (15, 16), *Raptorex* is known from an articulated skeleton of a subadult or young adult approximately 5 to 6 years old, with an adult body length of no more than 3 m (16).

Many features place *Raptorex* within Tyrannosauroidae, a clade including Tyrannosauridae and their closest relatives (Fig. 4). In the skull, for example, the premaxilla is anteroposteriorly short and bears teeth with incisiform crowns, the maxilla has only 13 teeth, the intermaxillary suture is fused, the jugal has a marked inflection along its ventral margin, and the retroarticular process of the lower jaw is short and transversely broad (Fig. 1, A and B, and D to F). In the postcranial skeleton, likewise, the scapula has a strap-shaped blade, and the forelimb and ilium are relatively short and long, respectively. The principal phylogenetic question, rather, is where *Raptorex* falls within Tyrannosauroidae.

The skull is proportionately large as in tyrannosaurs, measuring approximately 40% of trunk length (Fig. 2A). The skull in most other theropods, including the basal tyrannosauroid *Guanlong* (8), is relatively smaller, measuring about 30% or less of trunk length (16). Many additional cranial characters link *Raptorex* and large-bodied tyrannosaurs. The functional significance of some of these characters is poorly understood, such as the textured, highly vascularized nasals (also in *Eotyrannus*), the sutural contact between the lacrimal and frontal along the orbital margin, the pneumatic invasion of the bodies of the lacrimal and squamosal, or the enlarged surangular foramen (Fig. 1, A and B) (16).

Strengthening of the skull roof, on the other hand, is clearly the functional role of another suite of advanced cranial characters linking *Raptorex* and large-bodied tyrannosaurs (17, 18). The nasals are more strongly transversely arched (Fig. 1D) than in the basal tyrannosauroids *Dilong* (5) or *Xiongguanlong* (9), and the sutural surface between the nasal and maxilla is corrugated as in tyrannosaurs (3, 19) rather than planar. The antorbital fenestra, in addition, is proportionately short and lacks any nasal contribution to its dorsal margin, as in tyrannosaurs (3, 19, 20).

The functional role of another suite of advanced cranial characters linking *Raptorex* and large-bodied tyrannosaurs involves the expansion of attachment sites for jaw-closing (adductor) musculature. The supratemporal fossae in *Raptorex* extend broadly onto the frontals and meet along a median sagittal crest, which is particularly prominent anteriorly (Fig. 1, B and D). Attachment site expansion for adductor musculature also includes a flange on the dorsal aspect of the squamosal and a prominent surangular shelf at the posterior end of the lower jaw (Fig. 1A).

Differentiation of a set of smaller, incisiform teeth at the anterior end of the upper jaw is another functional specialization shared by *Raptorex* and tyrannosaurs. The last premaxillary tooth

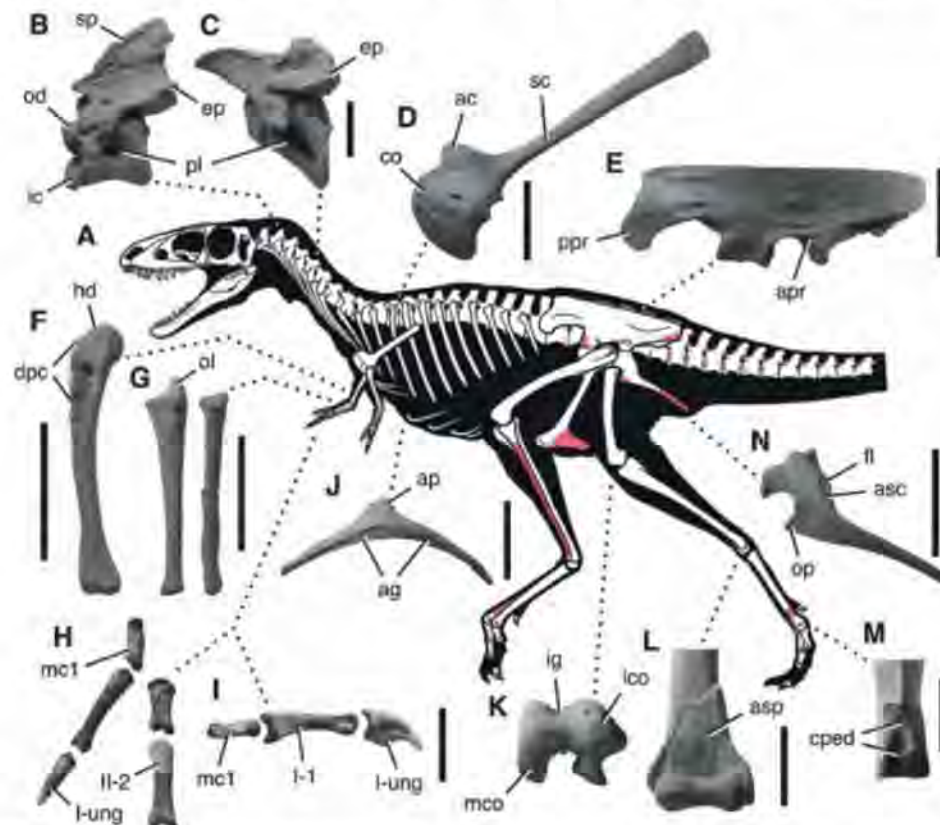
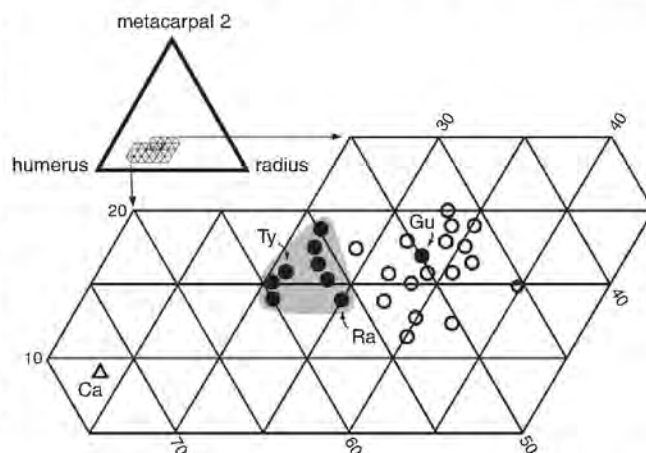


Fig. 2. Postcranial features of the Early Cretaceous tyrannosauroid *R. kriegsteini*. (A) Skeletal silhouette showing preserved bones (missing portions shown in red; the cast of the bones was used to eliminate color distractions). (B) Axis (C2) in left lateral view. (C) Midcervical vertebra (C5) in left lateral view. (D) Scapulocoracoid (left) in lateral view. (E) Ilium (left) in lateral view. (F) Humerus (left) in anterior view. (G) Ulna and radius (left) in medial view. (H) Manus (right) in dorsal view. (I) Manual digit I (right) in medial view. (J) Co-ossified anterior gastral elements in ventral view. (K) Femur (left) in distal view. (L) Distal tibia and astragalus (left) in anterior view. (M) Distal metatarsal 3 (right) in posterior view. (N) Proximal ischium (left) in lateral view. Abbreviations: l-1, manual digit I phalanx 1; l-ung, manual digit I ungual; ll-2, manual digit II phalanx 2; ac, acromion; ag, articular surface for successive gastralia; ap, anterior process; apr, acetabular process; asc, attachment scar; asp, ascending process; co, coracoid; cped, condylar pedicle; dpc, deltopectoral crest; ep, epipophysis; fl, flange; hd, head; ic, intercentrum; ig, intercondylar groove; mc1, metacarpal 1; mco, medial condyle; lco, lateral condyle; od, odontoid; ol, olecranon; op, obturator process; pl, pleurocoel; ppr, pendant process; sc, scapula; sp, spine. Scale bars, 2 cm in (B), (C), (H), and (I); 3 cm in (M); 5 cm in (D), (F), (G), and (J) to (L); 10 cm in (E) and (N).

Fig. 3. Ternary morphospace plot for principal forelimb segments (humerus, radius, and metacarpal 2) as percentages of total forelimb length in nonavian theropods, showing *Raptorex* near *Tyrannosaurus* and other short-armed tyrannosaurs (gray tone) and *Guanlong* among non-tyrannosaurid theropods with relatively unreduced forelimbs. Open circles are nontyrannosaurid theropods; solid dots are tyrannosauroids; the open triangle is the abelisauroid *Carnotaurus*. The three nontyrannosaurid theropods (open circles) closest to the tyrannosauroid cluster are coelophysoids (*Coelophysis* and *Syntarsus*). Data are from (2, 8, 27). Abbreviations: Ca, *Carnotaurus*; Gu, *Guanlong*; Ra, *Raptorex*; Ty, *Tyrannosaurus*.



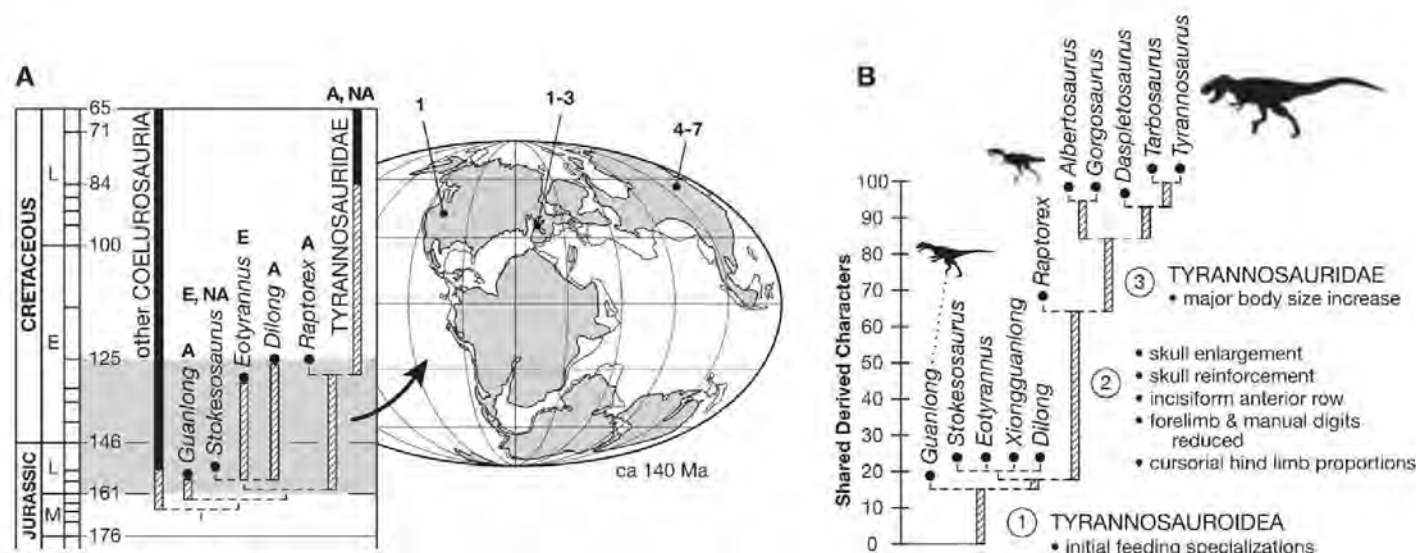


Fig. 4. Temporal, geographic, and phylogenetic patterns among tyrannosauroids. **(A)** Temporally calibrated phylogeny of tyrannosauroids based on phylogenetic analysis (16), showing an early diversity of basal tyrannosauroids (shaded) from localities across Laurasia and plotted on an Early Cretaceous paleogeographic map (28). *Proceratosaurus* (13) and *Xiongguanlong* (9) were excluded from this plot because of limited available morphologic data and age uncertainty, respectively. **(B)** Phylogram scaled to the amount of character change (delayed transformation) based on

cladistic analysis of 101 characters in 11 tyrannosauroids (consensus of 28 minimum-length trees of 123 steps; consistency index = 0.862, retention index = 0.954) (16). Circled nodes 1 to 3 outline major transformations in tyrannosauroid evolution. Taxonomic definitions (1) of Tyrannosauroidae, Tyrannosauridae, and other taxa follow (29). Abbreviations for biogeographic area and localities: A, Asia; E, Europe; NA, North America; 1, *Stokesosaurus*; 2, *Proceratosaurus* and *Eotyrannus*; 3, *Aviatyrannis*; 4, *Guanlong*; 5, *Dilong*; 6, *Xiongguanlong*; 7, *Raptorex*.

is substantially smaller than the first maxillary tooth, and all four premaxillary crowns are incisiform (with a D-shaped cross section) with a posteriorly facing median ridge (Fig. 1, A, E, and F). In the basal tyrannosauroids *Guanlong* (8) and *Dilong* (5), in contrast, tooth size is gradational to the maxillary series, and only the anterior two premaxillary teeth have a D-shaped cross section and a discrete posterior ridge (16). The maxillary crowns in *Raptorex*, nevertheless, remain transversely compressed as in other basal tyrannosauroids, unlike the stout, subcylindrical crowns in mature tyrannosaurids (20).

A partial endocast shows enlarged, semi-circular olfactory bulbs with a volume of approximately 2.5 cm³ situated adjacent to one another in the midline (Fig. 1C). The olfactory bulbs are 60% of the maximum width of the cerebral hemispheres and nearly 20% of their volume, which is significantly larger than in other nonavian coelurosaurs (21, 22) but resembles the enlarged condition in tyrannosaurids (23). The swollen cerebral hemispheres each have a volume of approximately 14.0 cm³, which is approximately 60% of the hemispherical volume in *Allosaurus* (24), a theropod with a body mass (~1000 kg) at least 10 times that of *Raptorex* (~60 to 100 kg, comparable to *Deinonychus*) (24, 25). Thus, it appears that the cerebrum in *Raptorex*, as in tyrannosaurids and other nonavian coelurosaurs, is relatively large. *Raptorex*, however, lacks the long olfactory stalk and undivided cerebrum of tyrannosaurids, which are best interpreted as size-related features arising independently in large-bodied theropods (22).

The vertebral column can be partitioned into 10 cervical, 13 dorsal, and 5 co-ossified sacral

vertebrae, as well as the first 11 caudal vertebrae of the tail (Fig. 2A) (16). The vertebral column is not a skeletal division that exhibits marked modification in tyrannosauroids, and *Raptorex* and other basal tyrannosauroids show few modifications particular to tyrannosaurids. The cervical centra are opisthocelous and have relatively longer centra and lower spinous processes than in tyrannosaurids (Fig. 2, B and C). A pneumatopore (pleurocoel) is present on the sides of all presacral centra and most of the sacra, as in tyrannosaurids and in contrast to the more limited axial pneumaticity in *Guanlong* (8). The gastral cuirass (stomach ribs), in contrast to the vertebrae, show modifications found only in tyrannosaurids. The anteriormost segment, which is composed of fused medial elements, has a subtriangular anteromedian process and a posterior articular groove for contact with the next pair of gastral elements (Fig. 2J) (2).

The scapulocoracoid has a prominent acromial process and narrow, strap-shaped blade, closely resembling the condition in tyrannosaurids as compared to the basal tyrannosauroids *Guanlong* and *Dilong* (Fig. 2D). The diminutive forelimb, likewise, is remarkably similar to that in tyrannosaurids and unlike the longer forelimb of *Guanlong*, which resembles that in other basal coelurosaurs such as *Ornitholestes*. The humerus has a sub-spherical head and reduced deltopectoral crest, and the ulna has a prominent olecranon process, straight shaft, and flat distal end (Fig. 2, F and G). The partially preserved manus also shows advanced features shared with tyrannosaurids, in contrast to that in *Guanlong* and *Dilong*, such as the reduction of metacarpal 1 (length subequal to the first phalanx of the digit II, medial distal

condyle rudimentary) and lengthening of the first phalanx of digit I (Fig. 2H). The small size of the first metacarpal and the very close correspondence in form of this metacarpal and the preserved manual phalanges to those in tyrannosaurids (2) suggest that the manus was probably functionally didactyl.

The relative length and proportions within the forelimb of *Raptorex* correspond well with those in tyrannosaurids. Humeral length is 29% that of the femur in *Raptorex* and tyrannosaurids, which is substantially shorter than in the basal tyrannosauroids *Guanlong* (63%) and *Dilong* (53%) (Table 1). Within the forelimb, the humerus is longer relative to either the radius or metacarpus, constituting nearly 60% of forelimb length rather than 50% as in *Guanlong*. Proportional variation within the forelimb is best visualized on a ternary plot summarizing the percentage contribution to forelimb length of the humerus, radius, and metacarpal 2 (Fig. 3). *Raptorex* plots near a cluster of tyrannosaurids with distinctive forelimb proportions, whereas *Guanlong* plots in the middle of an array of nontyrannosaurid, nonavian theropods.

The pelvic girdle of *Raptorex* exhibits derived features shared with tyrannosaurids that are absent in other basal tyrannosauroids such as *Guanlong* (8), *Stokesosaurus* (11), *Dilong* (5), and *Xiongguanlong* (9). The elongate ilium has a straight dorsal margin that appears to be pressed against the sacral neural spines, and a marked antitrochanter is present on the supraacetabular shelf, which does not project laterally beyond the ischial peduncle (Fig. 2E). The ischium exhibits a prominent rugose flange for muscle attachment and a narrow tapering shaft (Fig. 2N), and the

Table 1. Skull and long-bone lengths (in centimeters, upper portion) and proportions (in %, lower portion) of *R. kriegsteini* and other tyrannosauroids (2, 5, 8, 30). Parentheses indicate estimates; dashes indicate missing data. Measurements average long-bone lengths when both sides are available.

Measure or ratio	<i>Guanlong</i> IVPP V14531	<i>Dilong</i> IVPP V14243	<i>Raptorex</i> LH PV18	<i>Albertosaurus</i> AMNH 5664	<i>Tyrannosaurus</i> FM PR2081
Skull	36.3	16.6	(30.0)	67.8	139.4
Humerus	26.3	9.6	9.9	20.5	38.5
Radius	17.8	—	5.2	10.0	17.3
Metacarpal 2	8.9	—	(2.4)‡	6.0	10.4
Femur	41.6	18.0	33.8	70.0	131.5
Tibia	42.4	19.9	39.7	74.8	114.3
Metatarsal 2	34.0	11.2	24.5	(41.8)§	58.4
Metatarsal 4	36.0	11.1	26.6	(44.6)§	62.1
Humerus/femur	63%	53%	29%	29%	29%
Humerus/forelimb*	50%	—	56%	56%	58%
Radius/forelimb	33%	—	30%	27%	26%
Metacarpal 2/forelimb	17%	—	14%	16%	16%
Tibia/femur	102%	111%	118%	107%	87%
Femur/hindlimb†	35%	37%	34%	37%	43%
Tibia/hindlimb	35%	40%	40%	40%	37%
Metatarsal 4/hindlimb	30%	23%	26%	23%	20%

*Forelimb length equals the sum of the humerus, radius, and metacarpal 2. †Hindlimb length equals the sum of the femur, tibia, and metatarsal 4. Metatarsal 4 is used because only the distal end of metatarsal 3 is preserved in *Raptorex*. ‡Estimated from metacarpal 1, based on the metacarpal ratio in *Tyrannosaurus* FM PR2081 (2). §Estimated from metatarsal 3, based on the metatarsal ratio in *Tyrannosaurus* FM PR2081 (2).

pubis has a distal foot with a prominent anterior ramus (Fig. 2A).

The hindlimb in *Raptorex* also exhibits several derived features and proportions shared with tyrannosauroids. The femoral anterior trochanter extends proximally as far as the greater trochanter, the femoral distal condyles are deeply divided fore and aft (Fig. 2K), and the ascending process of the astragalus is tall (Fig. 2L). Metatarsal 3 is wedge-shaped (arctometatarsalian) proximally and has a raised nonarticular platform adjacent to the condyles distally (Fig. 2M). Within the hindlimb, tibial length is 118% that of the femur, a longer proportion than has been recorded in any other tyrannosauroid (Table 1).

Phylogenetic analysis of Tyrannosauroidae (16) (Fig. 4) confirms the basal position of *Guanlong*, *Stokesosaurus*, *Eotyrannus*, *Dilong*, and *Xiongguanlong* relative to *Raptorex* and Tyrannosauridae (5, 8–10). Although a few synapomorphies support *Guanlong* as the basalmost tyrannosauroid and *Xiongguanlong* as closer to *Raptorex* and Tyrannosauridae, relationships among these five basal tyrannosauroids remain poorly resolved and collapse with one additional step (Fig. 4B). Many derived features, in contrast, provide strong support for positioning *Raptorex* as the sister taxon to a monophyletic Tyrannosauridae. With only a few autapomorphies evident in its skeletal anatomy (15), *Raptorex* closely approximates a hypothetical ancestor on the lineage leading to Tyrannosauridae.

Three major morphological stages can now be visualized in the evolutionary history of Tyrannosauroidae (Fig. 4B). The first stage includes tyrannosauroids of Middle Jurassic to Early Cretaceous age with a trans-Laurasian dis-

tribution that are of small to medium body size and exhibit some initial feeding specializations. They include the Middle Jurassic genus *Proceratosaurus* (13); the Late Jurassic genera *Guanlong* (8), *Stokesosaurus* (11), and *Aviatyrannis* (12); and the Early Cretaceous genera *Eotyrannus* (10), *Dilong* (5), and *Xiongguanlong* (9) (Fig. 4A, shading). Femoral length, a rough proxy for body size, varies from 18 cm in *Dilong* (5) to 67 cm in *Stokesosaurus* (11) and is always less than that among subadult or adult tyrannosauroids (approximately 70 to 130 cm) (Table 1). Diagnostic tyrannosauroid features in this first stage involve initial strengthening of the snout via internasal fusion, specialization of the anteriormost upper teeth as incisors, and initial enlargement of the attachment area for jaw-closing musculature. In most other respects, the functional specializations seen in tyrannosauroids are lacking in the most complete of these early tyrannosauroids (5, 8, 10, 11).

The second stage involves the most conspicuous functional specializations of tyrannosauroids: a proportionately large skull with accessory pneumatization, a set of incisiform premaxillary teeth more distinctive in size and form, expanded jaw-closing musculature operating jaws with fewer teeth, a diminutive forelimb with distinctive intralimb proportions and joint morphology, and an elongate hindlimb with cursorial proportions and a fully developed compact-splint (arctometatarsalian) metatarsus (Fig. 4, node 2). All are manifest in *Raptorex*, a possible contemporary of the more primitive tyrannosauroid *Dilong*, from Lower Cretaceous rocks dating to about 125 Ma—some 40 million years before the oldest known tyrannosauroid (Fig. 4A).

The third stage, comprising currently known tyrannosauroids, is characterized by a marked increase in body size (Fig. 4, node 3). Some features, such as the more robust cheek teeth with subcylindrical cross section, distinguish tyrannosauroids from other large theropods such as spinosaurids and carcharodontosaurids. Many of the now more limited set of derived features that characterize tyrannosauroids, however, may owe their appearance to size increase, such as bony flanges within the orbit and laterotemporal openings, the prominence of occipital and vertebral processes for attachment to cervical musculature, the relative shortening of vertebral length, and less cursorial limb proportions. Adult body length in *Raptorex* is 3 m or less, corresponding to a body mass of approximately 65 kg, based on estimates for similar-sized theropods such as *Velociraptor*, *Deinonychus*, and *Oviraptor* (16, 25, 26). Tyrannosauroids, in contrast, range in body length from 6 to 12 m, corresponding to a body mass range of 2500 to 6000 kg (16, 25, 26). Body mass thus increased at least 40-fold from that in *Raptorex* to basal tyrannosauroids and eventually more than 90-fold to that in *Tyrannosaurus*.

Raptorex, in sum, reveals that the tyrannosauroid morphotype—the oversized skull, muscle-bound jaws, tiny forelimbs, and fleet-footed hindlimbs—evolved at modest body size some 125 million years ago (Fig. 4A). These features, singly or in concert, can no longer be explained as a passive allometric consequence of body size increase or the product of an extended (peramorphic) growth trajectory (5, 14). Instead, these features seem first to have evolved as an efficient predatory strategy at relatively small body size. It remains to be seen whether miniature precursors such as *Raptorex* eventually will be discovered for other large-bodied predatory radiations among dinosaurs, such as abelisauroids, spinosaurids (megalosauroids), and carcharodontosaurids. For tyrannosauroids, the predatory skeletal design embodied in *Raptorex* was scaled up with little modification in descendants with body mass as much as 90 times greater.

References and Notes

- We use the following phylogenetic definitions: **Tyrannoraptora**, the least inclusive clade containing *Tyrannosaurus rex* Osborn 1905 and *Passer domesticus* (Linnaeus 1758); **Tyrannosauroidae**, the most inclusive clade containing *Tyrannosaurus rex* Osborn 1905 but not *Ornithomimus edmontonicus* Sternberg 1933, *Troodon formosus* Leidy 1856, *Velociraptor mongoliensis* Osborn 1924; **Tyrannosauridae**, the least inclusive clade containing *Tyrannosaurus rex* Osborn 1905 and *Gorgosaurus libratus* Lambe 1914, *Albertosaurus sarcophagus* Osborn 1905.
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15. **Etymology:** *raptor*, plunderer (Greek); *rex*, king (Greek); *kriegsteini*, after Roman Kriegstein, in whose honor the specimen was secured for scientific study. **Holotype:** LH PV18, partially articulated skeleton composed of disarticulated cranial bones representing most of the skull and postcranial skeleton, lacking portions of the forelimb and the distal one-half of the tail (beyond the 11th caudal). The holotype represents a young adult, as shown by fusion of the nasals and braincase elements in the skull and at least partial fusion of all neurocentral sutures. Cataloged in the collection of the Long Hao Institute of Geology and Paleontology (Hohhot, Nei Mongol Autonomous Region) and the University of Chicago (Chicago). **Locality:** Approximately 41°20'N and 119°40'E, collected privately in the border area between Liaoning Province and the Nei Mongol Autonomous Region of the People's Republic of China. **Horizon and associations:** Lujiatun Beds of the Yixian Formation, comprising a tuffaceous fluvial facies of the Jehol Group with its well-known Jehol Biota that includes the teleost *Lycoptera* and pelecypods, which were found in association with the holotypic skeleton (16). The matrix around the fossil is light green, massive, poorly sorted, tuffaceous, micaceous sandstone with fibrous gypsum.
- The light-colored, uncrushed bones were buried for the most part in articulation. The absence of laminated, fine-grained sediment or conchostracans characterizes the Lujiatun Beds of the Yixian Formation, dated to the late Early Cretaceous (Barremian-Aptian, ~125 Ma) (16). **Diagnosis:** Basal tyrannosauroid with a narrow accessory pneumatic fossa within the antorbital fossa dorsal to the maxillary fenestra, jugal suborbital ramus of particularly narrow depth (transverse width approximately 60% of vertical depth), and absence of a vertical crest on the iliac blade dorsal to the acetabulum.
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31. For final drafts of all figures, we thank C. Abraczinskas. For preparation of fossil material, we thank personnel of the Western Paleontological Laboratories and the Fossil Lab at the University of Chicago. For assistance with computed tomography imaging, we thank C. Straus and C. Wietholt; for preparation of paleohistologic sections, we thank E. Lamm and D. Varicchio. For examination of fossil material in their care, we thank X. Xu, M. Norell, and S. Hutt. Supported by the Whitten-Newman Foundation and the National Geographic Society (to P.C.S.). This is Publication 3 of the Chinese-American Dinosaur Expeditions.

Supporting Online Material

www.sciencemag.org/cgi/content/full/1177428/DC1

Materials and Methods

Figs. S1 to S8

Tables S1 and S2

References

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Deep-Sea Archaea Fix and Share Nitrogen in Methane-Consuming Microbial Consortia

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Nitrogen-fixing (diazotrophic) microorganisms regulate productivity in diverse ecosystems; however, the identities of diazotrophs are unknown in many oceanic environments. Using single-cell-resolution nanometer secondary ion mass spectrometry images of ^{15}N incorporation, we showed that deep-sea anaerobic methane-oxidizing archaea fix N_2 , as well as structurally similar CN^- , and share the products with sulfate-reducing bacterial symbionts. These archaeal/bacterial consortia are already recognized as the major sink of methane in benthic ecosystems, and we now identify them as a source of bioavailable nitrogen as well. The archaea maintain their methane oxidation rates while fixing N_2 but reduce their growth, probably in compensation for the energetic burden of diazotrophy. This finding extends the demonstrated lower limits of respiratory energy capable of fueling N_2 fixation and reveals a link between the global carbon, nitrogen, and sulfur cycles.

Nitrogen-fixing (diazotrophic) bacteria and archaea convert dinitrogen (N_2) into ammonia (NH_3) for assimilation. Biological N_2 fixation counteracts the removal of bioavailable N by microbial processes such as denitrification and anaerobic ammonium oxidation (anammox) and provides a source of N to the majority of the biosphere that cannot directly assimilate N_2 . Many photosynthetic cyanobacteria fix N_2 in ocean surface waters and have been the primary focus of studies on marine

diazotrophy. Recently, a discrepancy between the calculated rates of oceanic denitrification and N_2 fixation has suggested that other less well-studied or currently unknown diazotrophic microorganisms may exist and fix substantial amounts of N_2 (1–5). Indeed, recent discoveries of new phylogenetically and physiologically diverse diazotrophs, including hyperthermophilic methanogens from hydrothermal vents (6), have shown that N_2 fixation can occur in extreme environments and localized habitats of enhanced productivity in the deep sea (5, 7, 8).

Here we show that syntrophic aggregates of archaea (of the ANME-2 group) and bacteria [*Desulfosarcina/Desulfococcus* (DSS)] mediating sulfate-dependent anaerobic oxidation of methane (CH_4) (AOM) in deep-sea sediments are

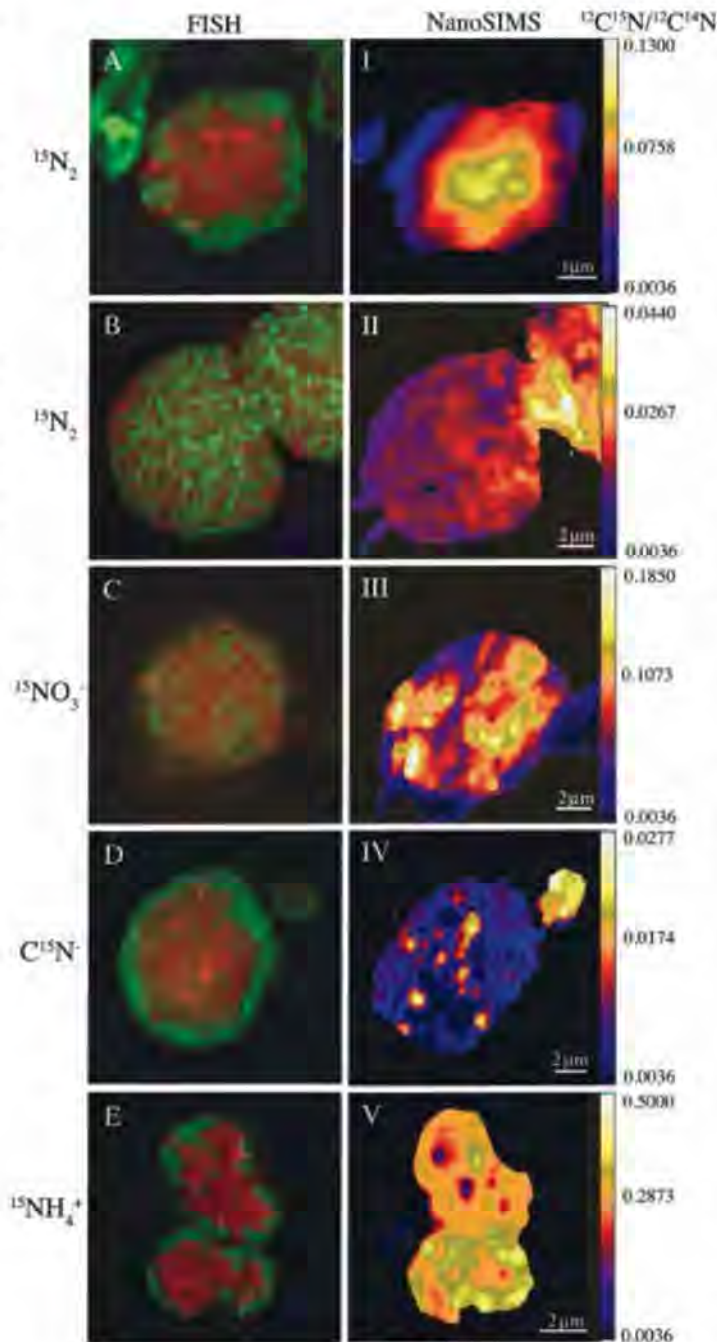
capable of N_2 fixation. The ANME-2/DSS consortia have been studied in recent years both because of their potentially critical role in marine carbon cycling and their enigmatic obligate syntrophy (9, 10). These consortia are most abundant in areas of high CH_4 concentration, such as cold seeps, but are present throughout continental margin sediments [(9) and references therein]. They currently represent the main filter for oceanic CH_4 release to the atmosphere, consuming up to 80% of naturally released CH_4 in marine sediments (9); however, the specific mechanism(s) coupling the ANME-2 and DSS cells remains unclear. Recent metagenomic sequencing of the ANME-2/DSS consortia identified the presence of nitrogenase genes required for N_2 fixation (*nif* genes) (11). This result, along with preliminary N isotope data, suggests that microbes within the consortia are able to fix N (11). We used submicron-scale ion imaging by nanometer secondary ion mass spectrometry (nanoSIMS) coupled to fluorescence in situ hybridization (FISH) to specifically identify the ANME-2 species as diazotrophs while detailing and quantifying patterns of N assimilation within the individual members of these metabolically interdependent consortia.

Sediment samples from an active CH_4 seep in the Eel River Basin, California, USA, were collected and anaerobically incubated with CH_4 and one of several ^{15}N -labeled N sources (12) (table S1). Nitrogen fixation, as demonstrated by the assimilation of ^{15}N from $^{15}\text{N}_2$ in coaggregated ANME-2 and DSS cells, occurred in all AOM consortia measured after 6 months of incubation with CH_4 (12) (Fig. 1, A and B, and Fig. 2A). ^{15}N enrichment within the consortia was as high as 10.5 ^{15}N atom %, which is 26 times the highest value observed in unlabeled ANME-2/DSS

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Fig. 1. Magnitude and distribution of ^{15}N incorporation in representative methanotrophic ANME-2/DSS consortia from sediments incubated with CH_4 and different ^{15}N -labeled N sources, as indicated. (A to E) FISH using probes targeting ANME-2 (Eel932) in red and *Desulfobacteraceae* (DSS658) in green. (I to V) Ion micrographs of $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$ ratios of the same microbial consortia imaged by FISH in (A) to (E), demonstrating the location of ^{15}N incorporation. The scale range varies between ion micrographs, with the minimum consistently set to natural abundance $^{15}\text{N}/^{14}\text{N}$.



consortia (ranging from 0.35 to 0.4 ^{15}N atom %). Inhibition of either CH_4 oxidation (incubations lacking CH_4) or sulfate reduction [incubations treated with the inhibitor sodium molybdate (Na_2MoO_4)] prevented ^{15}N incorporation (Fig. 2), implying that N_2 fixation requires a functioning symbiosis between the CH_4 -oxidizing ANME-2 and sulfate-reducing DSS partners. Other microbial cells from the $^{15}\text{N}_2$ incubation were not enriched in ^{15}N (maximum 0.38 ^{15}N atom %, $n = 10$ cells), suggesting that $^{15}\text{N}_2$ incorporation was specific to the ANME-2/DSS consortia over the incubation period and not due to nonspecific cycling of reduced ^{15}N after fixation by an unrelated group of organisms. ^{15}N was also incorporated from ^{15}N -labeled cyanide (C^{15}N^-), a toxic molecule structurally similar to

N_2 and known to be detoxified and assimilated by some, but not all, diazotrophs (13) (Figs. 1D and 2A). The broad substrate recognition by nitrogenase is hypothesized to be a relict ability from when the protein first evolved, when it may have primarily catalyzed reactions other than N_2 fixation [such as cyanide detoxification (12, 14–16)].

N_2 fixation appears to be primarily mediated by ANME-2, based on the distribution of ^{15}N within the consortia. In aggregates of shelled morphology (an inner sphere of archaeal cells surrounded by an outer layer of bacterial cells, approximately 500 cells total; $n = 5$ aggregates), the ^{15}N label was concentrated in the center of the aggregate, where the ANME-2 biomass was concentrated (Figs. 1A and 3). Addition-

ally, ANME-2/DSS aggregates showed ^{15}N enrichment colocalized with light $\delta^{13}\text{C}$ biomass, a signal diagnostic of methanotrophic ANME-2 species (11, 17) ($n = 6$ aggregates; fig. S1). This differs from the variable pattern of ^{15}N incorporation observed in the majority of aggregates from incubations amended with ^{15}N -labeled ammonium ($^{15}\text{NH}_4^+$) and nitrate ($^{15}\text{NO}_3^-$) and indicates that the elevated enrichment from $^{15}\text{N}_2$ within the ANME-2 archaea is attributable to diazotrophic activity, not simply a varying rate of protein synthesis between species (Fig. 1).

Serial FISH and SIMS images collected through individual aggregates reveal the three-dimensional distribution of ^{15}N assimilation from $^{15}\text{N}_2$ within AOM consortia (Fig. 3). The difference in ^{15}N atom % between the group of cells on the aggregate exterior (DSS-dominated) and the group in the interior (ANME-dominated) became greater with increasing penetration into the core of the aggregate, corresponding to an increasingly pure population of ANME in the interior (Fig. 3). Although the aggregate exterior averaged 31% less ^{15}N enrichment than the interior, all of the DSS cells on the periphery of the aggregate were enriched in ^{15}N relative to natural abundance [average ^{15}N atom % = 3.47 exterior ($n = 313$ regions of interest (ROIs)) and 5.01% interior ($n = 297$ ROIs), Fig. 3], suggesting a passage of reduced N from the ANME cells in the interior to the DSS-dominated exterior. The reduced ^{15}N enrichment in the DSS cells relative to the ANME cells is consistent with the trend observed in ^{15}N labeling studies of other symbioses, in which reduced N is shared between a diazotrophic and a nondiazotrophic partner (18, 19). Transfer of reduced N species between symbionts is common, often in exchange for energy-rich metabolites or structural protection (20). It is possible that inherent variations in metabolism and growth between the two partners may also lead to an offset in ^{15}N enrichment (21), and the possibility of concurrent fixation by both syntrophic partners at differing rates cannot be excluded at this time. However, in the context of molecular data acquired in parallel, this scenario appears less likely.

The analysis of *nif* sequences recovered from the $^{15}\text{N}_2$ sediment incubation was consistent with previous reports of a CH_4 seep-specific *nif*/H clade (fig. S2). The diverse *nif*/H genes recovered clustered primarily within a divergent clade of sequences reported from geographically distant deep-sea CH_4 seeps and whole-cell enrichments of ANME-2/DSS consortia from the Eel River Basin (11, 22) (fig. S2). The existence of this *nif*/H clade highlights the strong similarities between putative diazotrophs at geographically distant CH_4 seeps; however, its divergence from known diazotrophs has made previous attempts to assign the clade to either the Bacteria or Archaea speculative (22). We therefore collected and analyzed partial *nif* operons from the incubations and found that they contained the typical gene

Fig. 2. Effect of N source on rates of cellular growth and respiration. (A) ^{15}N incorporation in ANME-2/DSS consortia from bulk sediment incubated with each of the indicated N sources and/or inhibitors. Each data point represents the average ^{15}N (atom %) value of the most ^{15}N -enriched ion image (depth plane) of a single aggregate. Symbol size and shape indicate the time the aggregate was incubated: small solid black circles, 1 month; small gray circles, 3 months; large gray circles, 4 months; large black circles, 6 months. The horizontal dashed line represents natural abundance $^{15}\text{N}/^{14}\text{N}$. NM, not measured. (Inset) Change in abundance of aggregates (aggs) over time in the N_2 (dashed line) and NH_4^+ (solid line) incubations determined by staining with 4',6'-diamidino-2-phenylindole. Error bars represent 1 SD from the mean. (B) Sulfide production in the different ^{15}N sediment incubations. Each data point (square symbol) represents the value for a single incubation bottle at the time sampled, following the same symbol size and color trend noted in (A). There was no inhibition by 2-bromoethanesulfonic acid (BES) (12).

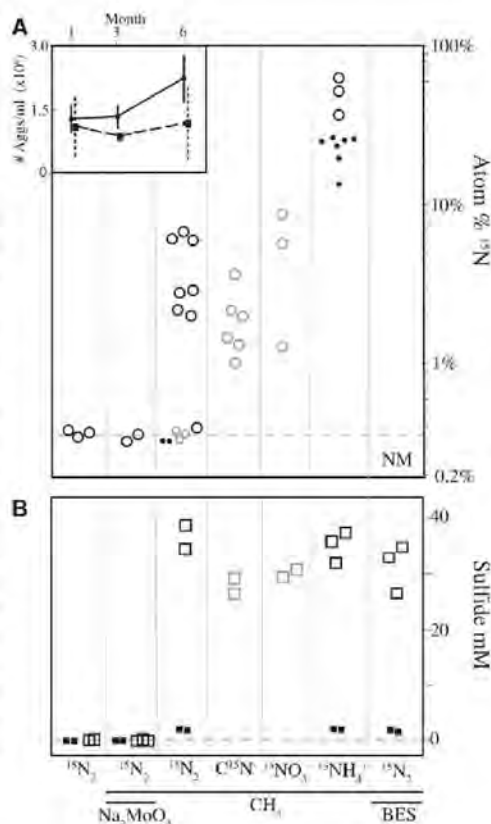
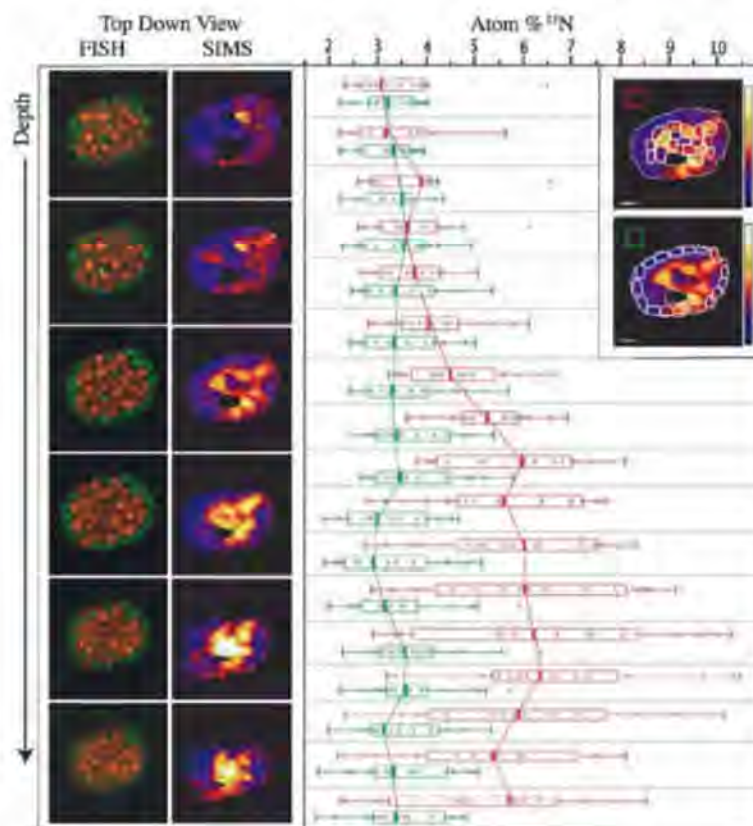


Fig. 3. Serial FISH and $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$ ion images in a representative shelled ANME-2/DSS aggregate showing the distribution of ^{15}N incorporation from $^{15}\text{N}_2$ with depth (left panels). In the FISH series, red indicates archaeal cells and green indicates bacteria. Comparison of ^{15}N incorporation on the inside of the aggregate (dominated by archaea) and the outside (dominated by bacteria) is shown on the right. Each gray data point represents the $^{15}\text{N}/^{14}\text{N}$ of a hand-drawn ROI, approximating the size of a cell ($1\ \mu\text{m}$) (12). Box and whisker plots indicate 75%, median, and 25% values for all ROIs drawn in either the inside (ANME-2, red) or outside (DSS, green) at a particular depth in the aggregate. The inset at right shows an example of ROIs designated for interior and exterior portions of the cell aggregate from a single depth. All ion micrograph values are scaled from 0.0036 to 0.11.



order (*nifH*, *nifI*, *nifD*, and *nifK*) of the C-type operon in methanogenic archaea and some nonproteobacterial anaerobic diazotrophs (23) (fig. S3). Additionally, the *nifD* phylotypes within these operons grouped within a well-supported clade containing sequences retrieved from other CH_4 seep sediment samples, methanogenic archaea (*Methanococcus*, 49% similarity), and nonproteobacterial N-fixing lineages rarely found at CH_4 seeps but which have been hypothesized to have undergone lateral gene transfer with archaea (such as *Clostridia* and *Roseiflexus* spp.) (23, 24) (Fig. 4). In the context of seep microorganisms, these data are most consistent with an archaeal origin for these operons. The *nifH* fragments of the partial operons cluster within the putatively seep-specific *nifH* clade, suggesting that this clade is archaeal, and supporting our designation of the ANME-2 archaea as the primary diazotrophic microorganism in the consortia.

N_2 fixation in ANME-2/DSS consortia is intriguing from an energetic standpoint; its cost is one of the highest for any anabolic process, requiring an investment of up to 16 adenosine triphosphate molecules (equivalent to $\sim 800\ \text{kJ}$) for each N_2 molecule reduced (8). Moreover, AOM coupled to sulfate reduction is thought to be one of the least energetically productive metabolisms known (10). At CH_4 seeps, coupled CH_4 oxidation and sulfate reduction reactions yield a total of approximately $40\ \text{kJ/mol}$ of CH_4 (10) that must be shared between the two syn-

trophic partners. Although other energy-limited diazotrophic microorganisms exist (such as methanogens) none to our knowledge generate less energy per mole of substrate than the ANME-2 species. One possibility is that in unusual environments, such as the deep sea, structural or mechanistic differences in the N_2 fixation machinery may alter the energetic burden. The low sequence similarity of the recovered *nif* genes to those previously described suggests some deviation from characterized N_2 -fixing systems (Fig. 4 and figs. S2 and S3).

Slowed growth is a common response to the energetic burden of N_2 fixation in active diazotrophs, including methanogenic archaea (25). Accordingly, using ^{15}N incorporation as a proxy for growth, the ANME-2/DSS consortia in this study actively fixing N grew approximately 20

times slower on average than aggregates grown in parallel with ammonium (Fig. 2). Although ANME-2/DSS growth rates are substantially affected by the available N source, the rate of AOM by the consortia [estimated by CH_4 -dependent sulfide production (12)] was similar during growth on either N_2 or NH_4 (Fig. 2B). Therefore, regardless of the exact amount of energy required to fix N_2 in these organisms, the consortia appear to compensate for the energetic burden of N_2 fixation by slowing growth while maintaining similar rates of respiration.

The maintenance of *nif* genes by the ANME-2 cells, and their consortial ability to fix N in the laboratory, imply that they do so in marine environments. Diazotrophy within deep-sea CH_4 seeps has not been detected directly, but N_2 fixation has been suggested at these locales,

based on low $\delta^{15}N$ values of seep sediment and fauna (26, 27). Why N_2 fixation would occur in anoxic marine sediments, often replete with ammonium, warrants further consideration. One explanation is that the CH_4 seep environment differs from typical anoxic sediment in that the main source of C (CH_4) is unaccompanied by N , poisoning its consumers for N limitation, similar to photoautotrophs (8) and aerobic methanotrophs (28). Indeed, measurements of pore water ammonium from the Eel River Basin CH_4 seeps were highly variable, ranging from 101 to 16 μM over a 6-cm sediment depth profile; these concentrations would not completely inhibit N_2 fixation in cultured diazotrophic methanogens (such as *Methanococcus maripaludis*) (29). Even in ammonium-replete sediments, localized zones of N limitation may occur (for example, within

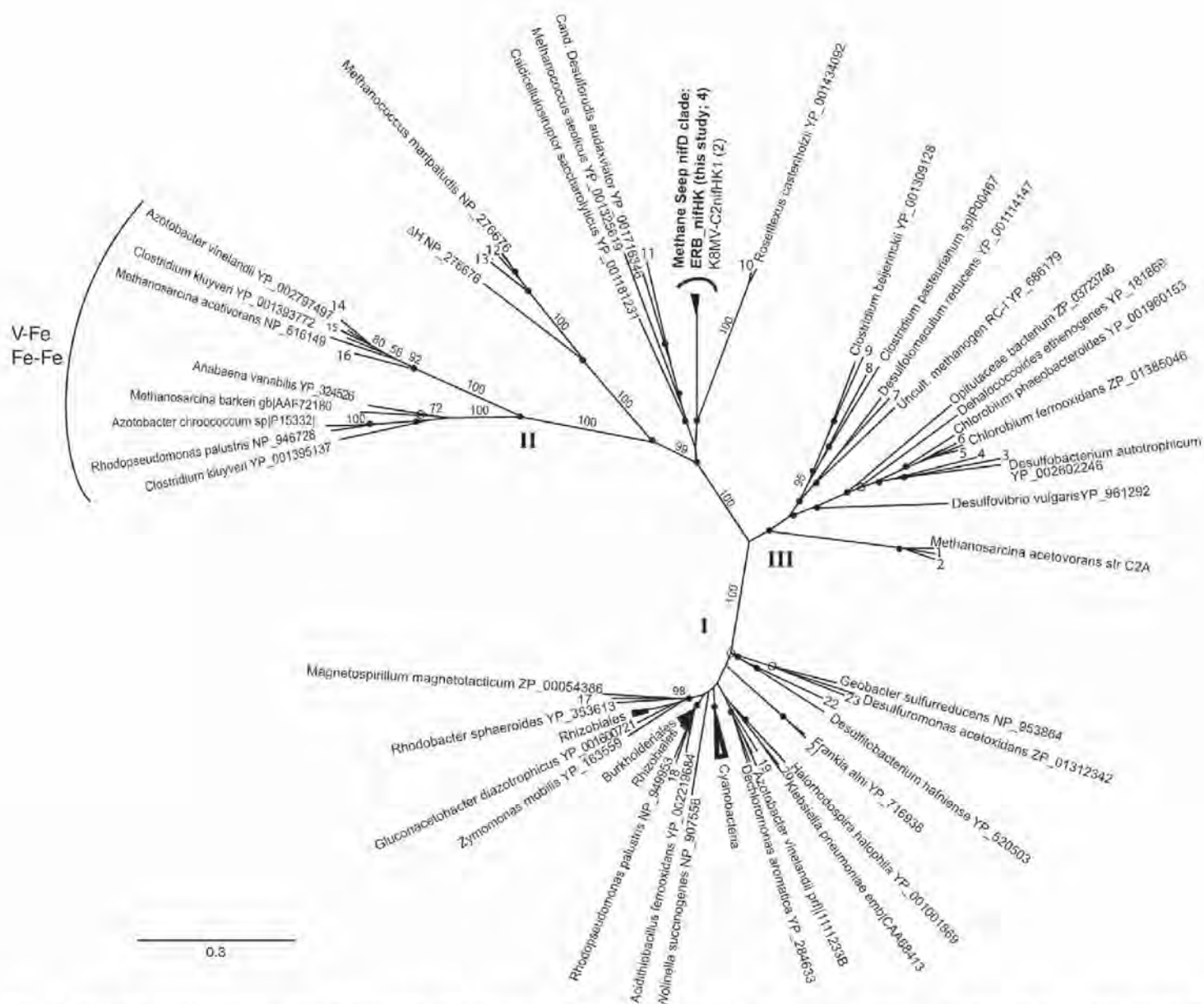


Fig. 4. Unrooted neighbor-joining tree of translated *nifD* sequences after global alignment. Bootstrap values from 85 to 100% (solid circles) and from 70 to 85% (open circles) are indicated at the nodes. The scale bar represents changes per amino acid position. The sequences obtained in this study are

shown in bold. Alternative nitrogenases are those that use V-Fe and Fe-Fe cofactors (*vnfD* and *anfD*, respectively). Roman numerals represent nitrogenase clusters as originally defined in (30). Names of sequences represented by numbers can be found in table S2.

densely packed microbial consortia). Although the loss of nitrate and ammonium from CH_4 seep sediments by catabolic bacterial processes (such as denitrification or anammox) has not yet been determined, these sinks for fixed N may also promote enhanced diazotrophy by the in situ microbial assemblage (3). Additionally, the current discrepancy in the oceanic fixed N budget underscores the possibility of new sources of fixed N in nontraditional and potentially unexpected habitats (1–3, 7). The extent to which the ANME-2/DSS consortia contribute to the putatively missing fraction of global fixed N inputs is unknown, but their input is probably not the only missing term in the equation. N_2 fixation in ANME-2, combined with the diversity of *nifH* genes recovered from marine sediments here and previously (5, 11, 22), suggests that our inventory of marine diazotrophs is incomplete and that we are only beginning to understand the extent and importance of benthic marine N_2 fixation.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/326/5951/422/DC1

Materials and Methods

SOM Text

Figs. S1 to S3

Tables S1 to S4

References

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Generation of Functional Ventricular Heart Muscle from Mouse Ventricular Progenitor Cells

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The mammalian heart is formed from distinct sets of first and second heart field (FHF and SHF, respectively) progenitors. Although multipotent progenitors have previously been shown to give rise to cardiomyocytes, smooth muscle, and endothelial cells, the mechanism governing the generation of large numbers of differentiated progeny remains poorly understood. We have employed a two-colored fluorescent reporter system to isolate FHF and SHF progenitors from developing mouse embryos and embryonic stem cells. Genome-wide profiling of coding and noncoding transcripts revealed distinct molecular signatures of these progenitor populations. We further identify a committed ventricular progenitor cell in the Islet 1 lineage that is capable of limited in vitro expansion, differentiation, and assembly into functional ventricular muscle tissue, representing a combination of tissue engineering and stem cell biology.

The mammalian heart is composed of a diversified set of muscle and nonmuscle cells that arise from multipotent progenitors in the first and second heart field (FHF and SHF, respectively) (1, 2). Defining the precise progenitor identity and the pathways that lead to ventricular myogenesis is critical for understanding cardiogenesis and also for regenerative cardiovascular medicine.

Accordingly, we generated a transgenic mouse with the red fluorescent protein dsRed under the control of an IsII-dependent enhancer of the

Mef2c gene whose expression is restricted to the SHF (3–5). We bred this mouse line with a transgenic mouse line in which enhanced green fluorescent protein (eGFP) is controlled by the cardiac-specific Nkx2.5 enhancer (6, 7). By fluorescence microscopy of double transgenic embryos on embryonic day 9.5 (E9.5), the entire primitive heart tube was eGFP+, but only the right ventricle (RV) and the outflow tract (OFT) were also dsRed+. Further, the pharyngeal mesoderm (PM), which contributes to the RV and OFT (8, 9), was dsRed+ but eGFP– (Fig. 1, A

to C). To delineate the in vivo expression of the reporters, we performed immunohistochemistry on E9.5 embryos and found that dsRed+/eGFP+ cells (R+G+) were restricted to the RV and OFT, dsRed–/eGFP+ cells (R–G+) to the left ventricle (LV) and inflow tract (IFT), and dsRed+/eGFP– cells (R+G–) to the PM (Fig. 1D).

Embryonic stem cell (ESC) lines make use of many of the in vivo developmental programs, providing an attractive model system for lineage commitment. Therefore, we generated multiple ESC lines that harbor both the Nkx2.5-eGFP and the SHF-dsRed reporters (fig. S1A). Fluorescence microscopy of chimeric embryos from these ESC lines revealed faithful recapitulation of marker expression (fig. S1, B and C). In vitro differentiation by embryoid body (EB) formation resulted in discrete populations of R+G+, R+G–, and R–G+ cells by EB day 6 (fig. S1D).

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To isolate ESC-derived FHF and SHF progenitor cells, we dissociated day 6 EBs into single-cell suspension and fluorescence-activated cell sorting (FACS)-purified four distinct populations

of cells: (i) R+G+, (ii) R+G-, (iii) R-G+, and (iv) unlabeled (R-G-) (Fig. 2A and fig. S2A). We then performed DNA microarray analysis on coding and noncoding RNA. Hierarchical

clustering (10) showed distinct reproducible expression patterns for the different cardiac progenitor subsets of mRNAs, as well as microRNAs (miRNAs) (figs. S3 to S5 and table S1). Next, we

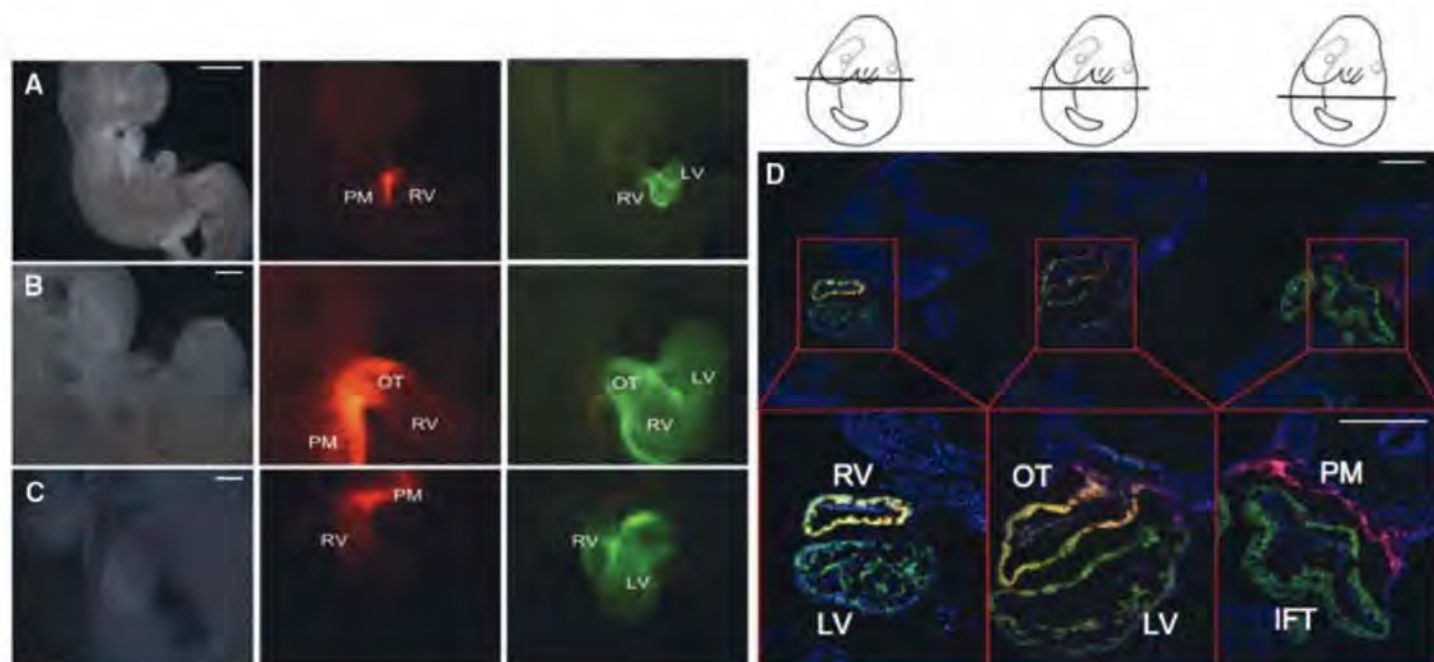


Fig. 1. E9.5 SHF-dsRed/Nkx2.5-eGFP transgenic embryos. (A to C) Whole-mount fluorescence microscopy of double transgenic E9.5 embryos. The LV and IFT are eGFP+ only, the RV and OT are dsRed+ and eGFP+, and the PM is dsRed+. Right lateral [(A) and (B), with (B) as close-up view] and left lateral (C) views are shown. Scale bar, 500 μ m (A);

100 μ m [(B) and (C)]. (D) Immunofluorescence labeling of E9.5 double transgenic mouse embryo shows that cells in the RV and OT are R+G+, in the PM are R+G-, and in the LV and IFT are R-G+. Section levels are shown as a cartoon. eGFP, green; dsRed, red; overlay, yellow. Scale bar, 50 μ m.

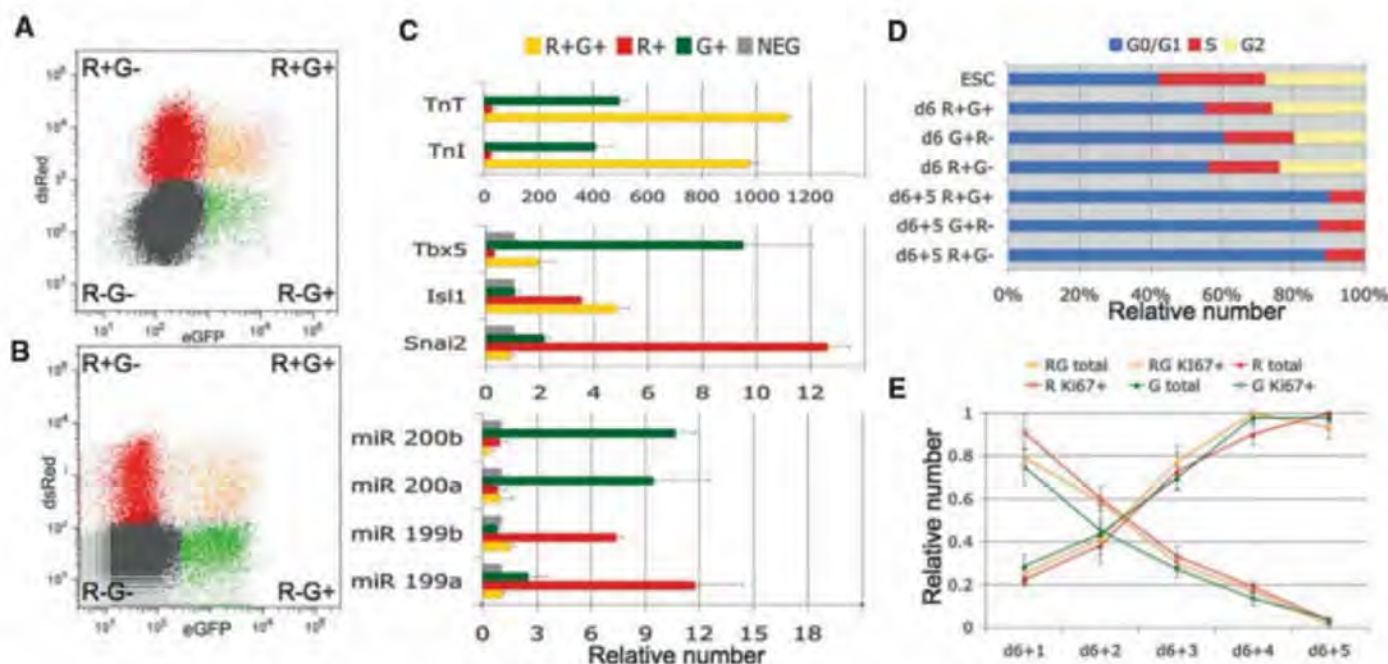


Fig. 2. Isolation of cardiac progenitors. (A and B) Representative flow cytometry plots of double transgenic day 6 EBs (A) and E9.5 embryos (B) showing four populations of cells: (i) R+G+, (ii) R+G-, (iii) R-G+, and (iv) R-G-. (C) QPCR analysis of mRNA and miRNA isolated from embryonic progenitors. Values are normalized against an unlabeled control. Error bars indicate SD. Differences between groups were highly statistically significant

(see tables S2 and S3). (D) Hoechst staining and FACS-based cell cycle analysis of undifferentiated ESCs, EB day6 progenitors, and differentiated progeny. (E) ESC-derived cardiac progenitors were cultured for 5 days. 4',6'-diamidino-2-phenylindole and Ki67 staining was performed to quantify the total cell number and proportion of cycling cells (Ki67+ cells/total cells). Error bars indicate SD ($n = 4$).

FACS-purified E9.5 embryonic progenitors (Fig. 2B and fig. S2B). Quantitative real-time fluorescence polymerase chain reaction (QPCR) analysis on 100 mRNAs and 10 miRNAs revealed that ESC- and embryonic-derived progenitors, isolated immediately after FACS sorting, displayed similar but nonidentical patterns of expression (Fig. 2C and fig. S6). mRNAs and miRNAs implicated in cardiac development and disease were enriched in the colored cells compared with in unlabeled cells. Isl1, a marker for the SHF, was appropriately enriched in only the R+G+

and the R+G- populations, whereas T-box transcription factor 5 (*Tbx5*), a marker of the FHF (11, 12), was appropriately enriched only in the R-G+ population. The R+G+ cells appeared to resemble more closely the myogenic population based on the expression of myocardial markers such as cardiac troponins, cardiogenic transcription factors, and bone morphogenetic protein signaling molecules. Further, the R+G- population of the PM expressed high levels of *Snai2*, a transcription factor regulating epithelial-to-mesenchymal transition (EMT) and necessary for cell migra-

tion (13, 14), suggesting that SHF/PM progenitors undergo EMT before migrating during cardiogenesis. In addition, miRNA199a/b were preferentially expressed in the R+G- population, and miRNA200a/b in the R-G+ population, and may therefore be considered cardiac markers for the SHF and FHF, respectively (fig. S7).

Because a hallmark of progenitor cells is their capacity for expansion before differentiation, we performed Hoechst staining and FACS-based cell cycle analysis on undifferentiated ESC, EB day 6

Fig. 3. Differentiation potential of cardiac progenitors. Embryonic- and ESC-derived cardiac progenitors were cultured on fibronectin-coated slides (fibronectin) or micropatterns for 5 days. (A and B) Representative immunofluorescence microscopy of embryonic (A) or ESC-derived (B) progenitors cultured on micropatterns are shown. Nuclei, blue; smMHC, red; and sarcomeric α -actinin, green. Scale bar, 40 μ m. (C and D) Cell counting was used to quantify the relative number of CM (sarcomeric α -actinin positive) or SM (smMHC positive) derived from embryonic (C) or ESC (D) progenitors. R+G+ populations resulted in the most CM ($P < 0.001$). No significant differences were observed in SM differentiation ($P = 0.38$ to 1.0). P values for the differences in CM differentiation are displayed. Error bars indicate SD ($n = 4$).

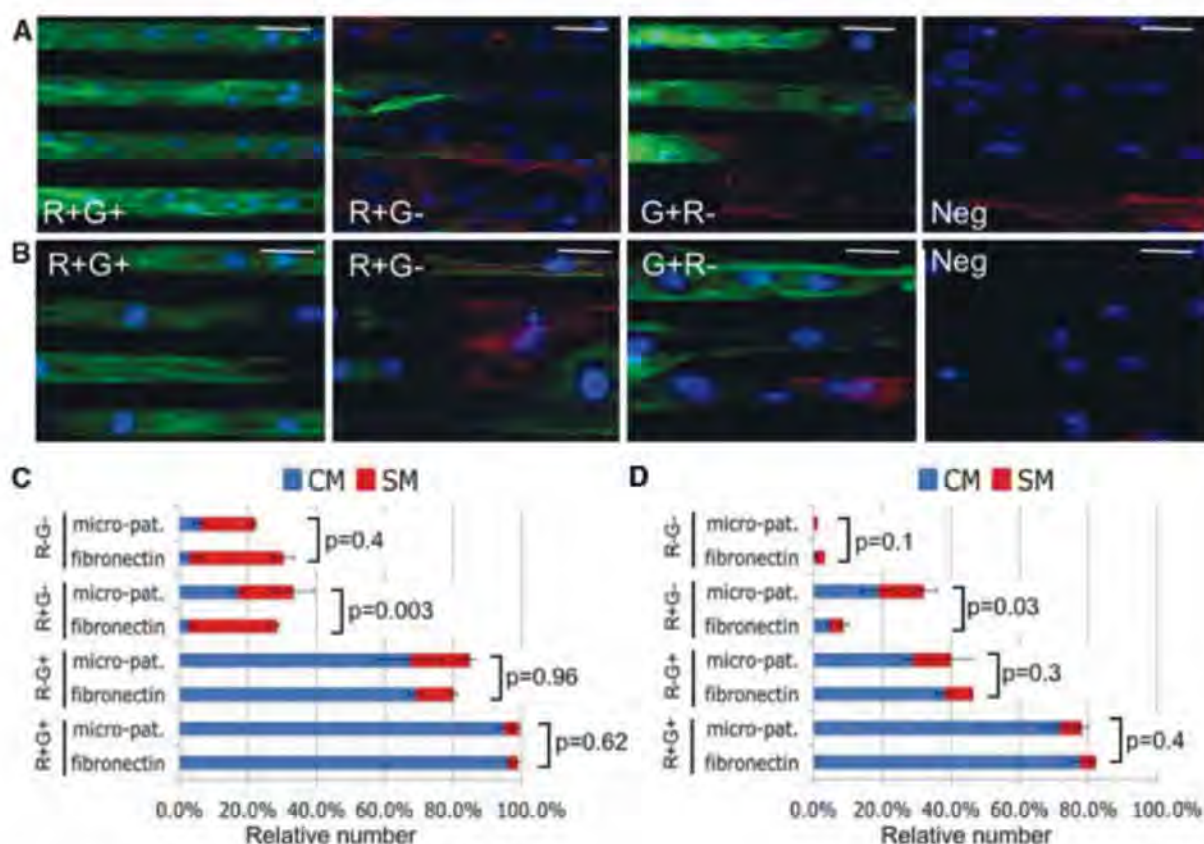
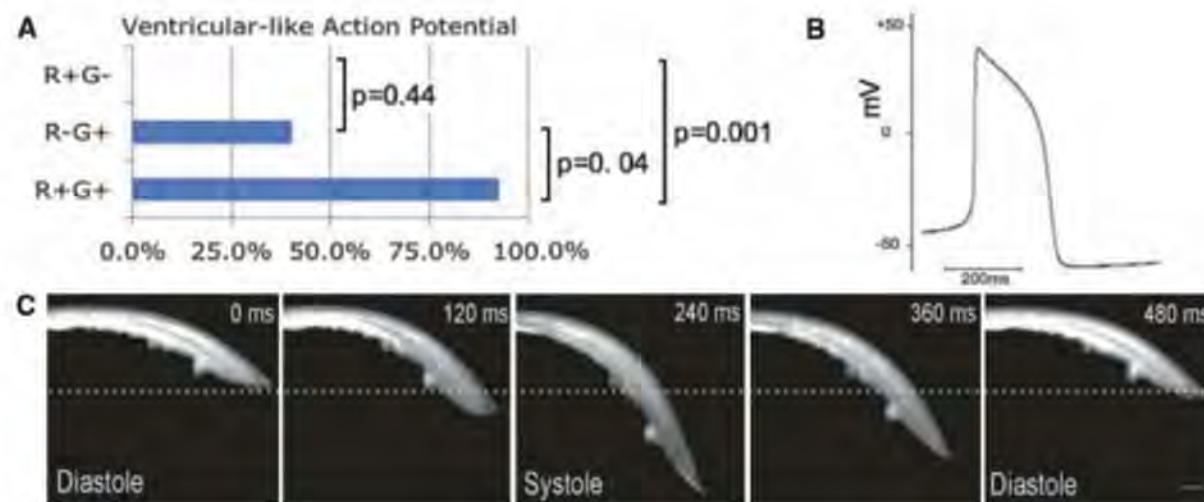


Fig. 4. Engineered ventricular tissue from R+G+ progenitors. (A) R+G+ ($n = 12$), R+G- ($n = 5$), and R-G+ ($n = 5$) progenitors were allowed to differentiate, and single-cell patch clamp recordings were performed. AP morphology was assessed for typical four-phase ventricular AP. (B) Representative spontaneous AP from R+G+ derived cardiomyocytes. (C) ESC-derived R+G+ progenitors were used to generate MTF as described in the SOM and in (15). Field stimulation (10 V, 10-ms pulse width) induced a 0.5-Hz cyclical contraction and rhythmic MTF bending (see movie S1).



cardiac progenitors, and their differentiated progeny (d6+5). ESC and EB day 6 progenitors had 40 to 55% of cells in combined S and G₂ phase, but the differentiated progeny had less than 15% of cells in S phase with none in G₂ phase (Fig. 2D). For validation, we isolated EB day 6 progenitors and allowed them to expand in vitro for an additional 5 days. Immunostaining with Ki67, a marker for actively cycling cells, showed that 24 hours after isolation, most cells were actively cycling, but this activity decreased over 5 days. Conversely, total cell number increased fourfold over the same 5-day period (Fig. 2E). Furthermore, the expression of the progenitor markers *Isl1* or *Tbx5* was maximal at the time of progenitor isolation but decreased with further differentiation (fig. S8). In contrast, Troponin T expression continued to increase with differentiation (fig. S8). Thus, the progenitor populations have a real but limited in vitro expansion potential. The drop off in expansion is concomitant with differentiation and loss of progenitor marker expression, suggesting that an endogenous clock may limit their proliferative capacity.

To examine progenitor myogenic potential, we cultured embryonic- and ESC-derived progenitors on either fibronectin-coated slides or micropatterns of 20- μ m-wide lines of fibronectin alternating with 20- μ m-wide lines of Pluronic F127 (a surfactant that blocks cell adhesion). After 5 days of in vitro expansion and differentiation, we performed immunofluorescence staining for sarcomeric α -actinin and smooth muscle myosin heavy chain (smMHC), labeling cardiomyocytes and smooth muscle, respectively. Plating embryonic- and ESC-derived R+G+ cells on micropatterned surfaces resulted in anisotropic tissue consisting of longitudinally aligned myocardial fibers (Fig. 3, A and B). In contrast, plating the progenitor populations on unpatterned slides resulted in isotropic unaligned tissue (fig. S9). Cell counting showed that embryonic- and ESC-derived R+G+ progenitors primarily gave rise to cardiomyocytes independent of surface culture conditions. In contrast, the R+G- and R-G+ populations gave rise to a more heterogeneous population of both smooth muscle and cardiomyocytes (Fig. 3, C and D). It remains unclear whether these cells represent homogenous populations of multipotent progenitors or heterogeneous populations of unipotent progenitors. Culturing R+G- (but not other) progenitors on micropatterned surfaces resulted in a statistically significant increase in the proportion of cardiac myocytes, suggesting that this population's myogenic potential may be modulated by microenvironmental geometric cues. Single-cell patch clamp experiments demonstrated that R+G+ progenitors differentiated into ventricular cardiac myocytes with typical four-phase action potential (AP), whereas R+G- and R-G+ progenitors differentiated into more heterogeneous cell types (Fig. 4, A and B; fig. S10; and table S4). Further, R+G+ cardiomyocytes showed sodium-

channel dependency, consistent with ventricular APs (fig. S11).

Next, we used the R+G+ progenitors to engineer two-dimensional cardiac tissue into a muscular thin film (MTF), as described in the supporting online material (SOM) and (15). The MTF beat spontaneously at a rate of ~20 contractions per minute and could be paced by field stimulation at 0.5 and 1.0 Hz. To measure contractility, the MTF was fixed as a cantilever on one end, and the contracting cardiomyocytes bent the MTF toward the cell side during systole (Fig. 4C and movie S1). During diastole, the elastic polydimethylsiloxane film provided the antagonistic force that returned the MTF back to the relaxed position. The change in radius of curvature is inversely proportional to cardiomyocyte stress generation and was measured at ~5 kPa for the progenitor-derived cardiac tissue at peak systole (fig. S12), similar to MTFs engineered from neonatal rat ventricular cardiomyocytes (15).

This development of an in vivo multicolor reporter system in embryos and ESC lines now allows for the purification of distinct subsets of the earliest heart field progenitors. Whereas *Isl1* primarily marks the SHF (4, 16–18), there have been no distinct markers for the FHF lineages that contribute to the LV. Here we document distinct transcriptional signatures for the FHF and SHF lineages, including the expression of unique subsets of miRNAs, which suggest that these lineages have distinct identities. The identification of independent FHF markers should allow a rigorous analysis of their role in heart development and disease.

A critical step in cardiogenesis is the formation and expansion of the ventricular myocyte lineage. The discovery and purification from embryos and corresponding ESC lines of committed ventricular progenitors (CVPs) uncovers a mechanistic pathway for organogenesis through limited expansion and assembly of CVPs into fully functional ventricular muscle tissue. Thus, directed differentiation from multipotent islet progenitors to a specific differentiated progeny occurs via the formation of transient committed intermediate progenitors that are destined to become specific cell types. This finding suggests a general paradigm for the conversion of multipotent islet progenitors to other differentiated cell types, such as endothelial or conduction system cells (fig. S13). Furthermore, recent work has now identified multiple *Isl1* intermediate progenitor populations in human embryonic hearts and human ESCs (19), suggesting that it may be possible to isolate self-expanding human ventricular progenitors.

A central challenge for cell-based therapy has been the identification of an optimal cell type to drive robust cardiac myogenesis. The ideal heart progenitor cell would be derived from a renewable cell source in sufficient quantities to drive clinically relevant levels of cardiac myogenesis. In addition, it would be critical to direct the dif-

ferentiation of progenitor cells into functional ventricular myocytes, instead of related lineages such as smooth muscle cells. The ability to generate functional ventricular MTF from these progenitor cells displaying limited expansion potential should allow the direct chemical screening of previously unknown molecular entities for therapeutic endpoints that can only be measured on intact muscle tissue, including force development and conduction velocity. With recent advances in the generation of induced pluripotent stem cells (20–22), it should now be possible to isolate patient- and disease-specific cardiac progenitors. The combination of tissue engineering technology with stem cell biology, therefore, represents an approach for the development of human models of human disease and a platform for drug discovery and design.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S13

Tables S1 to S4

References

Movie S1

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Generation of Medaka Fish Haploid Embryonic Stem Cells

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Haploid embryonic stem (ES) cells combine haploidy and pluripotency, enabling direct genetic analyses of recessive phenotypes in vertebrate cells. Haploid cells have been elusive for culture, due to their inferior growth and genomic instability. Here, we generated gynogenetic medaka embryos and obtained three haploid ES cell lines that retained pluripotency and competitive growth. Upon nuclear transfer into unfertilized oocytes, the haploid ES cells, even after genetic engineering, generated viable offspring capable of germline transmission. Hence, haploid medaka ES cells stably maintain normal growth, pluripotency, and genomic integrity. Mosaic oocytes created by combining a mitotic nucleus and a meiotic nucleus can generate fertile fish offspring. Haploid ES cells may offer a yeast-like system for analyzing recessive phenotypes in numerous cell lineages of vertebrates in vitro.

Haploidy, the presence of a single genome or chromosome set per cell, as in yeast (*1*), is a powerful system for genetic analyses of molecular events because any recessive mutations of essential genes will show a clear phenotype due to the absence of a second gene copy. Embryonic stem (ES) cells are pluripotent and thus able to differentiate into almost all cell types, providing an excellent system for experimental analyses of cellular and developmental events in vitro (*2*). To date, diploid ES cells containing two chromosome sets have been derived from early fertilization embryos (*3–5*). Induced pluripotent stem cells can also be obtained from differentiated cells by forced expression of pluripotency transcription factors (*6*). Haploid ES cells combining both haploidy and pluripotency would hold enormous potential in biomedicine for analyzing recessive and disease phenotypes in various cell lineages in vitro. However, haploidy in

vertebrates exists only in the postmeiotic germline (*7*), and it is unknown whether haploidy can support stable growth (constant proliferation without crisis) and pluripotency in culture. Haploid cells in frog (*8*), *Drosophila* (*9, 10*), and human (*11*) were unstable and quickly overgrown by diploid and aneuploid cells in serial culture. Attempts in mice failed to detect any haploid mitoses in cultures from parthenogenetic haploid embryos (*12*). These data have led to a hypothesis that haploidy has inferior growth and genetic instability.

We envisioned that the generation of haploid ES cells might depend on three critical factors: (i) inbred strains free of deleterious alleles, (ii) conditions conducive to stem cell proliferation rather than differentiation, and (iii) the ability to retain genetic stability. These considerations led us to choose the fish medaka (*Oryzias latipes*) as a test organism. This fish is a useful model for vertebrate development (*13*). Previous work has generated diploid ES cells (*4, 14*), male germ stem cells (*15*), and many inbred strains permissive to ES cell derivation (*16*).

Medaka *i¹* and *i³* are inbred albino strains containing mutations in two different loci affect-

ing pigmentation. Their F1 hybrid shows wild-type (black) pigmentation (fig. S1A). Eggs of *i¹* were inseminated with ultraviolet-irradiated *i³* sperm, leading to haploid gynogenesis, development of embryos containing only a female genome without a sperm-supplied male genome (fig. S1, B and C). Stage-10 gynogenetic blastula embryos were dissociated into single blastomeres for feeder-free culture (*17*). Serial passages led to stably growing cultures (Fig. 1A). We initiated five primary cultures and obtained three haploid ES cell lines designated as HX1, HX2, and HX3, because they had stable growth and ES cell phenotype after more than 140 passages during more than 500 days of culture. Chromosome examination revealed that the three cell lines showed an average of 77% haploid cells, 18% diploid cells, and 5% polyploidy cells (table S1). Genotyping did not detect the sperm-derived autosomal tyrosinase allele (*14*) or Y-chromosomal *dmrt1y* in all the three cell lines (fig. S2). We conclude that the three cell lines predominantly comprise Haploid X-chromosome-carrying (hence the HX designation) cells of gynogenetic origin.

HX1, HX2, and HX3 exhibited similar growth and phenotype. They have an ES cell phenotype such as round or polygonal shape, little cytoplasm, and large nuclei with prominent nucleoli (Fig. 1, C to F), characteristics of diploid ES cells from fertilization-derived embryos of mouse (*5*), human (*3*) and medaka (*4*). When seeded at a low cell density, HX1 at 40 to 108 passages after 100 to 300 days of culture was able to form colonies (Fig. 1B). Colonies of undifferentiated cells steadily grew (Fig. 1, C to G) and produced pure clones (Fig. 1H) exhibiting high alkaline phosphatase activity (Fig. 1I), a general ES cell marker (*4*). Thus, these cell lines possess the characteristics of ES cells in continuous proliferation, colony formation, and phenotype.

To determine whether these haploid ES cells had the intrinsic ability for stable growth, we established individual cell clones again by clonal growth. Out of 100 random HX1 colonies, we

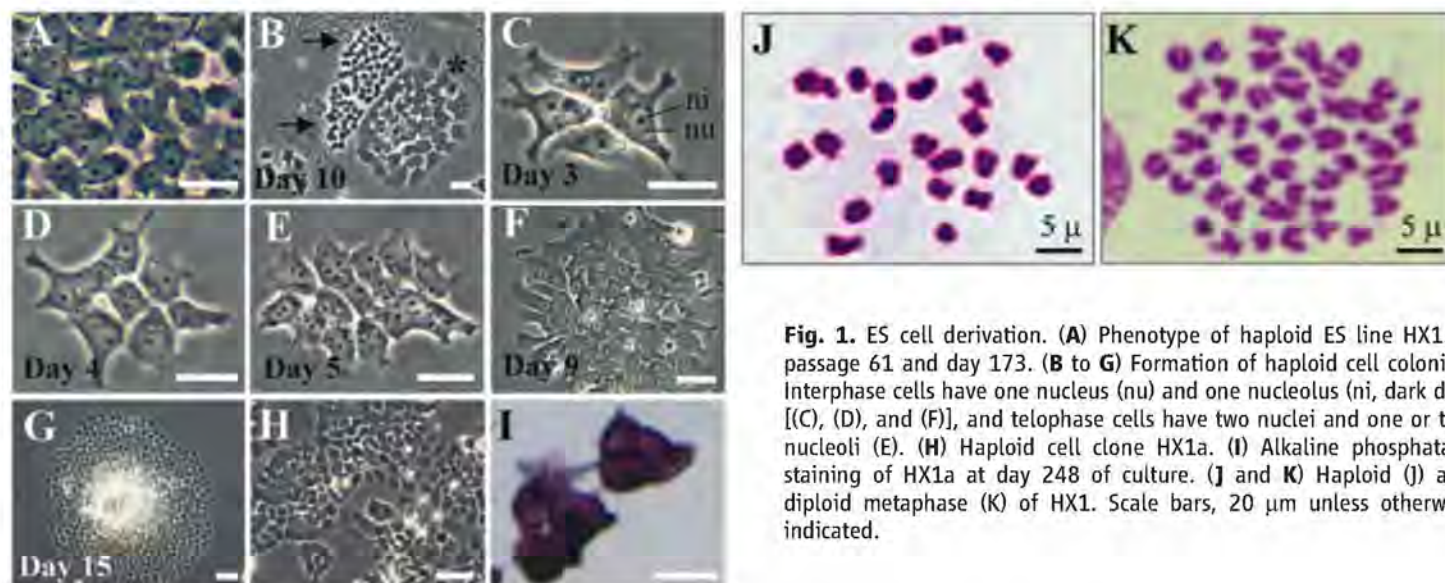


Fig. 1. ES cell derivation. (A) Phenotype of haploid ES line HX1 at passage 61 and day 173. (B to G) Formation of haploid cell colonies. Interphase cells have one nucleus (nu) and one nucleolus (ni, dark dot) [(C), (D), and (F)], and telophase cells have two nuclei and one or two nucleoli (E). (H) Haploid cell clone HX1a. (I) Alkaline phosphatase staining of HX1a at day 248 of culture. (J and K) Haploid (J) and diploid metaphase (K) of HX1. Scale bars, 20 μ m unless otherwise indicated.

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obtained 60 clones. Chromosome examination (table S2) revealed that 45 clones (75%) were haploid (Fig. 1J), with 15 (25%) being diploid (Fig. 1K), similar to the haploid-diploid ratio in parental cell cultures (table S1). Notably, haploid clones produced more haploid metaphases (84.6%) (tables S1 and S2). Flow cytometry

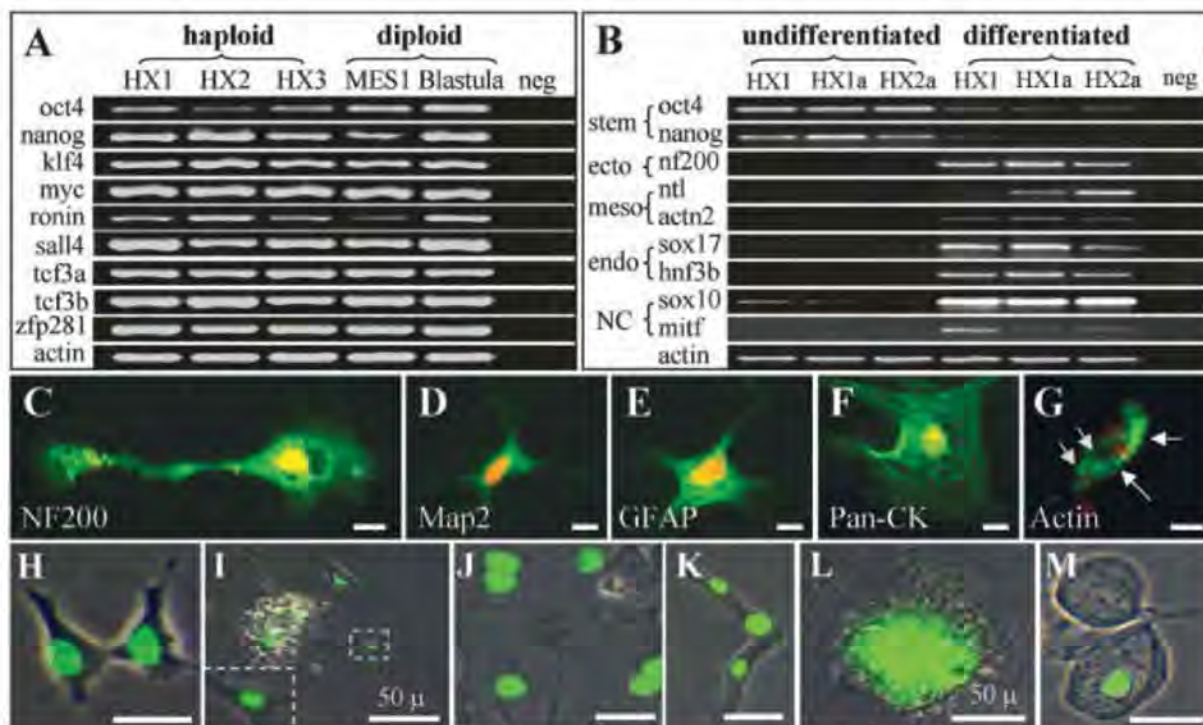
analyses revealed a major haploid peak and a minor diploid peak in the parental cell lines, but only a haploid peak in haploid clones (fig. S3). Collectively, HX1 clones comprise essentially pure haploid or diploid cells capable of stable growth.

Mouse and human ES cells specifically express pluripotency genes such as *oct4* and *nanog*

(6). We identified nine medaka homologs/paralogs of mammalian pluripotency genes by phylogenetic sequence analyses (fig. S4). We observed their expression in all three haploid cell lines, as in blastula embryos and diploid MES1 cells (Fig. 2A). Therefore, haploid cells retain pluripotency gene expression in culture.

Fig. 2. Pluripotency.

(A) Expression of pluripotency genes by reverse transcription polymerase chain reaction. Blastula, stage-10 embryos as positive control; MES1, diploid medaka ES line 1; neg, negative control without cDNA template. *actin* was used as loading control. **(B)** Expression of pluripotency genes (*oct4* and *nanog*) and differentiation genes in haploid ES cells after 10 days of EB formation. Stem, stemness; ecto, ectoderm; meso, mesoderm; endo, endoderm; NC, neural crest; HX1, parental HX1 line; HX1a and HX2a, haploid clones from HX1 and HX2, respectively. *nf200*, neurofilament 200; *ntl*, no tail; *actn2*, actinin 2; *hnf3b*, hepatocyte nuclear factor 3b; *mitf*, microphthalmia-associated transcription factor. **(C to G)** Immunostaining (green) and propidium iodide (red) nuclear staining of replated cells from 10-day-old EBs. (C) NF200-positive neurons. (D) Map2-positive neuron. (E) Glial fibrillary acidic protein-positive astrocyte. (F) PanCK-positive squamous epithelium. (G) Actinin2-positive matured muscle with



striations (arrows). **(H)** Nuclear GFP-HX1a cells. **(I to M)** Five-day-old chimera-derived HX1a derivatives in culture. **(I)** Neural cells with long cytoplasmic processes. **(J)** Fin epithelia. **(K)** Skeletal muscle with multiple nuclei. **(L)** Cardiomyocytes (also see movies S5 and S6). **(M)** Endodermal cells. Scale bars, 20 μ m unless indicated.

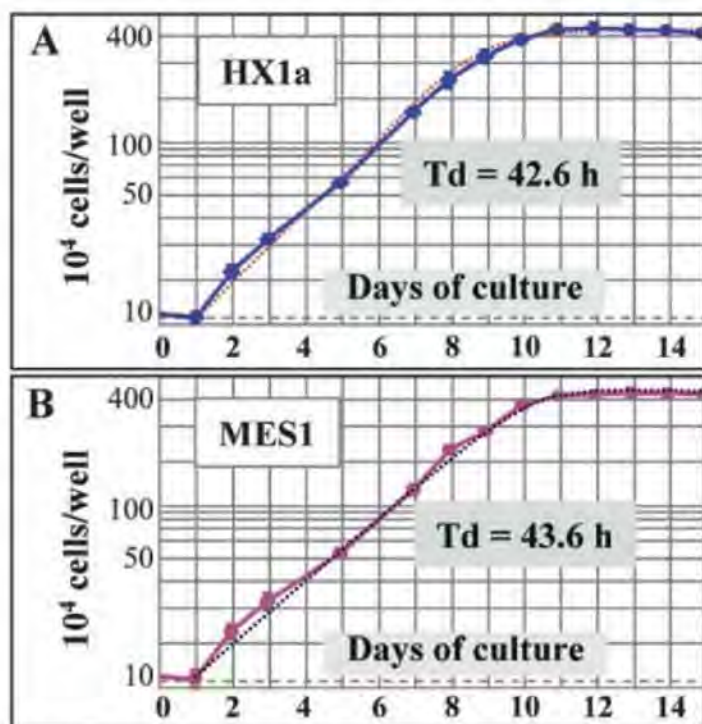


Fig. 3. Stable growth and ploidy. **(A and B)** Growth curves. Similar doubling time (T_d) is seen between haploid clone HX1a (A) and diploid MES1 (B). Triplicate counting was done for each time point. **(C to E)** Competitive clonal coculture. Haploid clones HX1a (green), HX2a [unlabeled; outlined in (E)] and diploid MES1 (red) formed distinct colonies. Scale bar, 20 μ m.

Diploid ES cells can form three-dimensional embryoid bodies (EBs) in suspension culture and manifest induced differentiation (3, 4). Under the culture condition for undifferentiated growth, MES1 and HX1a (a haploid clone of HX1) (table S2) underwent self-renewal (fig. S5, A and B). HX1a in suspension culture formed EBs (fig. S5C). In suspension coculture, HX1a and MES1 formed chimeric EBs (fig. S5D). In HX1a-derived EBs, decreased *oct4* and *nanog* expression accompanied activation and up-regulation of differentiation genes (Fig. 2B and fig. S5, E and F). These included genes specific to the ectoderm (*nf200*), the mesoderm [*actin2* and *brachyury* or *ntl* (18)], the endoderm (*sox17* and *hnf3b*) (19), and the neural crest (*sox10* and *mitf*) (20). Immunostaining of replated cell cultures from these EBs identified terminally differentiated cell types including neurons (Fig. 2, C and D), astrocytes (Fig. 2E), squamous epithelia (Fig. 2F), and striated muscle (Fig. 2G). Taken together, haploid ES cells are capable of lineage restriction and differentiation into derivatives of all the three germ layers in vitro.

We then tested pluripotency in vivo by chimerism formation (14, 16). Transplantation of HX1a cells at day 255 of culture into fertilization blastula hosts (fig. S6A) led to normal embryogenesis and 100% chimera formation ($n = 159$). HX1a cells distributed to all major compartments of early chimeric embryos at high efficiencies (60 to 90%) and many organs of the three germ layers (fig. S6,

B to F). HX1a cells generated terminally differentiated derivatives such as blood cells (fig. S6C and Movies S1 and S2) and contracting cardiomyocytes in the beating heart (fig. S6D and Movies S3 and S4). Cotransplanted HX1a and MES1 exhibited overlapping distribution in chimeric embryos (fig. S6, E and F) without cell fusion (fig. S6, G and H). HX1a was virtually indistinguishable from MES1 in distribution (fig. S6I). Evidence for terminal differentiation came from observations in cell cultures isolated from chimeric embryos. We found that nuclear green fluorescent protein (GFP)-labeled HX1a donor cells (Fig. 2H) were able to produce various cell types, including ectodermal neural cells (Fig. 2I) and fin epithelia (Fig. 2J), mesodermal skeletal muscles (Fig. 2K), and cardiomyocytes (Fig. 2L and Movies S5 and S6), as well as endodermal cells (Fig. 2M). Hence, HX1a is pluripotent in vivo.

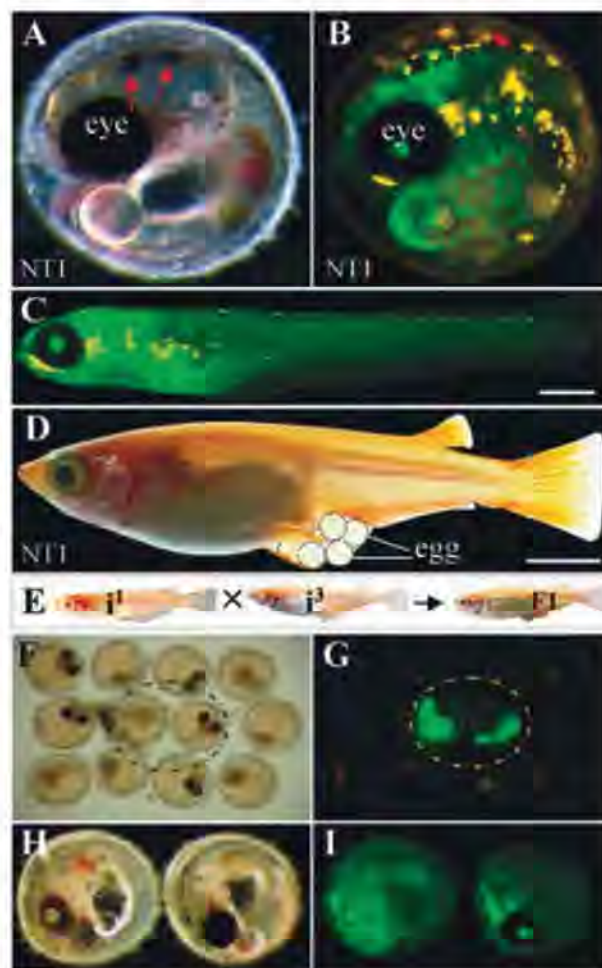
The difficulty of generating haploid cell cultures has been attributed to inferior growth and chromosomal instability (8–10). During serial passages under culture conditions for undifferentiated growth, the three cell lines maintained a constant haploid-diploid ratio (table S1), which suggests a comparable capability in cell division between haploidy and diploidy. We addressed this issue by three experiments. First, we used the nucleolar number as a marker to observe ploidy levels in living cells. Medaka cells have only one nucleolus per haploid genome (21). During the nucleolar

cycle (fig. S7A), a haploid medaka cell may have one (interphase), no (metaphase and anaphase), or two nucleoli (telophase with two daughter nuclei). The nucleolus is easily visible in living cells (Fig. 1, C to F), and its number delineates ploidy levels in culture. Nucleolar silver staining of fixed cells confirmed these observations (fig. S7B). Three percent of HX1 and MES1 cells lacked any visible nucleolus (fig. S7D), a characteristic of metaphase and anaphase cells, indicating a similarly high mitotic index. Cells with one nucleolus or two nucleoli increased from 50% in parental cell lines to 80% in haploid clone HX1a or diploid clone HX1b (fig. S7D), coinciding with essentially pure haploid or diploid ES cells by chromosome analysis (tables S1 and S2) and flow cytometry (fig. S3). Second, growth curves revealed a doubling time of 42.6 hours for haploid HX1a (Fig. 3A), comparable to 43.6 hours for diploid MES1 (Fig. 3B). Third, we analyzed two series of competitive growth assays. In the first series, we differentially labeled haploid clone HX1a and diploid MES1 and compared their growth in coculture and subculture. Mixed cells grew equally well into a monolayer, in which haploid and diploid cells formed distinct areas without detectable fusions (fig. S8A). Cell quantification revealed the retention of the initial haploid-diploid ratio during the entire coculture period of 30 days, up to 10 passages (fig. S8B). In the second series, GFP-labeled haploid ES clone HX1a, unlabeled haploid ES clone HX2a, and red fluorescent protein (RFP)-labeled diploid MES1 cells were seeded at a low density. They formed distinct colonies at a frequency relative to the initial ratio at seeding (Fig. 3, C to E). HX1 and its haploid clone HX1a generated three colony types at similar frequencies to those of MES1 (fig. S8C). Thus, haploid ES cells are not different from diploid ES cells in proliferation.

We analyzed the chromosomal stability in more detail. At a low density, steadily dividing, colony-forming cells were essentially haploid (fig. S9, A to C). We also observed bi- (fig. S9D) and multinucleated cells (fig. S9, E and F) apparently resulting from endomitosis by haploid cells. These bi- and multinucleated cells exhibited differentiated phenotypes and ceased proliferation (fig. S9, D to F). At a high density without subculture for 35 days, many cells fused into multinucleated skeletal muscles (fig. S9G). Thus, di- and polyploidization occurred in haploid ES cultures but did not increase during subsequent subcultures without overgrowing haploid ES cells. Consequently, the three parental cell lines maintained a minor proportion of polyploid metaphases (tables S1 and S2). Flow cytometry analyses revealed the absence of increased di- and polyploidized cells (fig. S3, C to H). Furthermore, both haploid and diploid clones exhibited stable growth and karyotype during subsequent clonal and massive culture (tables S1 and S2). Thus, the haploid cells retain chromosomal stability in vitro.

An assisted reproductive technology called semicloning (SC) has been proposed for treating infertile patients who lack germ cells (22). SC

Fig. 4. Generation of fertile nuclear transplant and germline transmission. (A and B) Nuclear transplant founder NT1 embryo at day 7. The i^1 -derived HX1a was stably labeled with nuclear GFP (Fig. 2H), and its nuclei were transplanted into nonenucleated oocytes of strain i^3 . Wild-type pigmentation is seen in the eye and body surface [arrows in (A)], and nuclear GFP is throughout the embryo (B). (C) Fry NT1 showing GFP expression and eye pigmentation. (D) Holly, NT1 fertile female laying eggs. (E) Crossing between albino strains i^1 and i^3 . F1 hybrids have wild-type black pigmentation in the eye. (F to I) Germline transmission. (F) and (G) Cluster of embryos between NT1 and i^1 male, showing germline transmission into four classes of progeny: GFP-positive albino or pigmented and GFP-negative albino or pigmented. (H) and (I) Large magnification of GFP-positive albino or pigmented progeny as circled in (F) and (G). Embryos are 1 mm in diameter. Scale bars, 0.2 mm (C) and 0.5 mm (D).



would use nuclear transfer to combine a haploid somatic nucleus (e.g., by somatic cell haploidization) from one parent and a haploid female or male gamete nucleus from the other parent into a mature oocyte. If a female gamete nucleus is used, a haploid somatic nucleus is directly introduced into a normal oocyte without enucleation. SC would allow for biparental contribution to the progeny and create a new and unpredictable combination of genetic traits from both parents similar to normal fertilization (22). We tested SC directly by producing mosaic oocytes in medaka. We labeled i^1 -derived HX1a cells (Fig. 2H) with nuclear GFP and transplanted their nuclei into nonenucleated mature oocytes of i^3 albino. Just as the F1 hybrid zygote from normal fertilization between these two albino strains produces wild-type pigmentation (fig. S1A), the mosaic oocyte from the SC procedure would also contain one HX1a-derived haploid i^1 nucleus and one i^3 oocyte nucleus and thus might generate offspring with black pigmentation and GFP expression. Out of 667 oocytes, 7 reached the hatching stage, and 3 hatched out to swimming fry (table S4). These nuclear transplants indeed exhibited black pigmentation and nuclear GFP in many tissues (Fig. 4, A to C). One of the nuclear transplant fry grew into a fertile female (Fig. 4D) that exhibited continuous GFP expression from the haploid ES genome and similar pigmentation to the fertilization hybrid between i^1 and i^3 albinos (fig. S1A and Fig. 4E), demonstrating the functional contribution from both the oocyte and HX1a nuclei, instead of meiotic genome duplication. We call this SC-derived fertile nuclear transplant Holly. Holly showed normal fertility and germline transmission upon test crosses to both i^1 and i^3 males, producing four types of F1 progeny: albino or pigmented and GFP-positive or -negative (Fig. 4, F to I). Pigmented and albino progeny were segregated at the Mendelian 1:1 ratio, albeit GFP-positive progeny represented only 23% (table S5). We raised the F1 animals to adulthood and examined their germline transmission again by test crosses. When crossed to nontransgenic i^1 fish, pigmented F1 animals heterozygous for GFP produced the same four different phenotypes in F2 generation. Female and male progeny from Holly were not different in exhibiting the 1:1 Mendelian segregation of pigmentation and GFP expression in F2 (table S6) and F3 generations (fig. S10), suggesting the absence of apparent parental defects. Because genomic abnormalities cannot support embryogenesis to advanced stages in mammals (22) and medaka (18), the production of Holly and its germline transmission demonstrates the retention of genetic stability and integrity in medaka haploid ES cultures. Hence, mosaic oocytes created by combining a haploid mitotic nucleus and a haploid meiotic nucleus can generate viable and fertile fish offspring. Holly and its progeny over three generations show normal embryonic and adult development (Fig. 4 and fig. S10). Uniparental diploid ES cell lines have been obtained from gynogenetic mammalian embryos (23). Whether

mammalian haploid ES cells could be generated and participate in a normal developmental program is unknown.

The lack of haploid human cell lines has led to alternative approaches such as using unstable near-haploid leukemia cultures (11) or human-rodent cell fusions to convert diploidy to haploidy (24). Featuring haploidy and pluripotency, the medaka haploid ES cell lines we obtained will provide a unique yeast-like system for directly analyzing recessive and disease phenotypes in various cell lineages of a vertebrate in vitro.

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Supporting Online Material

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Materials and Methods
Figs S1 to S10
Tables S1 to S6
Movies S1 to S6
References

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Complete Resequencing of 40 Genomes Reveals Domestication Events and Genes in Silkworm (*Bombyx*)

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A single-base pair resolution silkworm genetic variation map was constructed from 40 domesticated and wild silkworms, each sequenced to approximately threefold coverage, representing 99.88% of the genome. We identified ~16 million single-nucleotide polymorphisms, many indels, and structural variations. We find that the domesticated silkworms are clearly genetically differentiated from the wild ones, but they have maintained large levels of genetic variability, suggesting a short domestication event involving a large number of individuals. We also identified signals of selection at 354 candidate genes that may have been important during domestication, some of which have enriched expression in the silk gland, midgut, and testis. These data add to our understanding of the domestication processes and may have applications in devising pest control strategies and advancing the use of silkworms as efficient bioreactors.

The domesticated silkworm, *Bombyx mori*, has a mid-range genome size of ~432 Mb (1), is the model insect for the order Lepidoptera, has economically important values (e.g., silk and bioreactors production), and has been domesticated for more than 5000 years (2). Be-

cause of human selection, silkworms have evolved complete dependence on humans for survival (3), and more than 1000 inbred domesticated strains are kept worldwide (3). Archaeological and genetic evidences indicate that the domesticated silkworm originated from the Chinese wild silk-

worm, *Bombyx mandarina*, that is found throughout Asia, where modern sericulture and silkworm domestication were initiated.

The origin of the domesticated silkworm is a long-standing question that has not been settled by previous limited biochemical and molecular analyses. Two hypotheses suggested a unique domestication but disagreed on the ancestral variety.

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One hypothesis, based on isoenzyme polymorphism, proposed mono-voltinism as ancestral variety (voltinism represents number of generations per annum), from which bi- and multi-voltine were derived by artificial selection (4); the other proposed the reverse path considering evidence from archaeology, history, and genetics (5). An alternative hypothesis based on random amplification of polymorphic DNA indicated that the ancestral domestic silkworm strains were issued, not from a unique variety, but from mixed geographic locations and ecological types (6). These theories are conflicting, probably because they were derived from incomplete genetic information. Consequently, we present here a genome-wide detailed genetic variation map in hopes to help reconstruct the silkworm domestication history.

The data consisted of 40 samples from 29 phenotypically and geographically diverse domesticated silkworm lines [categorized by geographical regions (3): Chinese, Japanese, tropical, European lineages, and the mutant system], as well as 11 wild silkworms from various mulberry fields

in China (table S1). We sequenced each genome at approximately threefold coverage, after creating single- and paired-end (PE) libraries with inserts of PEs ranging from base pairs 137 to 307 (7).

Raw short reads were mapped against the refined 432-Mb reference genome from *Dazao* (1) with the program SOAP (8). We pooled all reads from the 40 complete genomes and identified 15,986,559 single-nucleotide polymorphisms (SNPs) using SoapSNP (7, 9) (table S3A). The accuracy of the SNP calling was evaluated with Sequenom (San Diego, California) genotyping of a representative subset of variants in all 40 varieties, resulting in a 96.7% validation rate (7).

We then pooled separately all 29 domesticated strains and all 11 wild varieties and obtained SNP sets for each (7). The number of SNPs in the domestic versus wild varieties was 14,023,573 and 13,237,865, respectively (table S3A). To account for the different number of domestic and wild strains, we used the population-size scaled mutation rate θ_s to measure genetic varia-

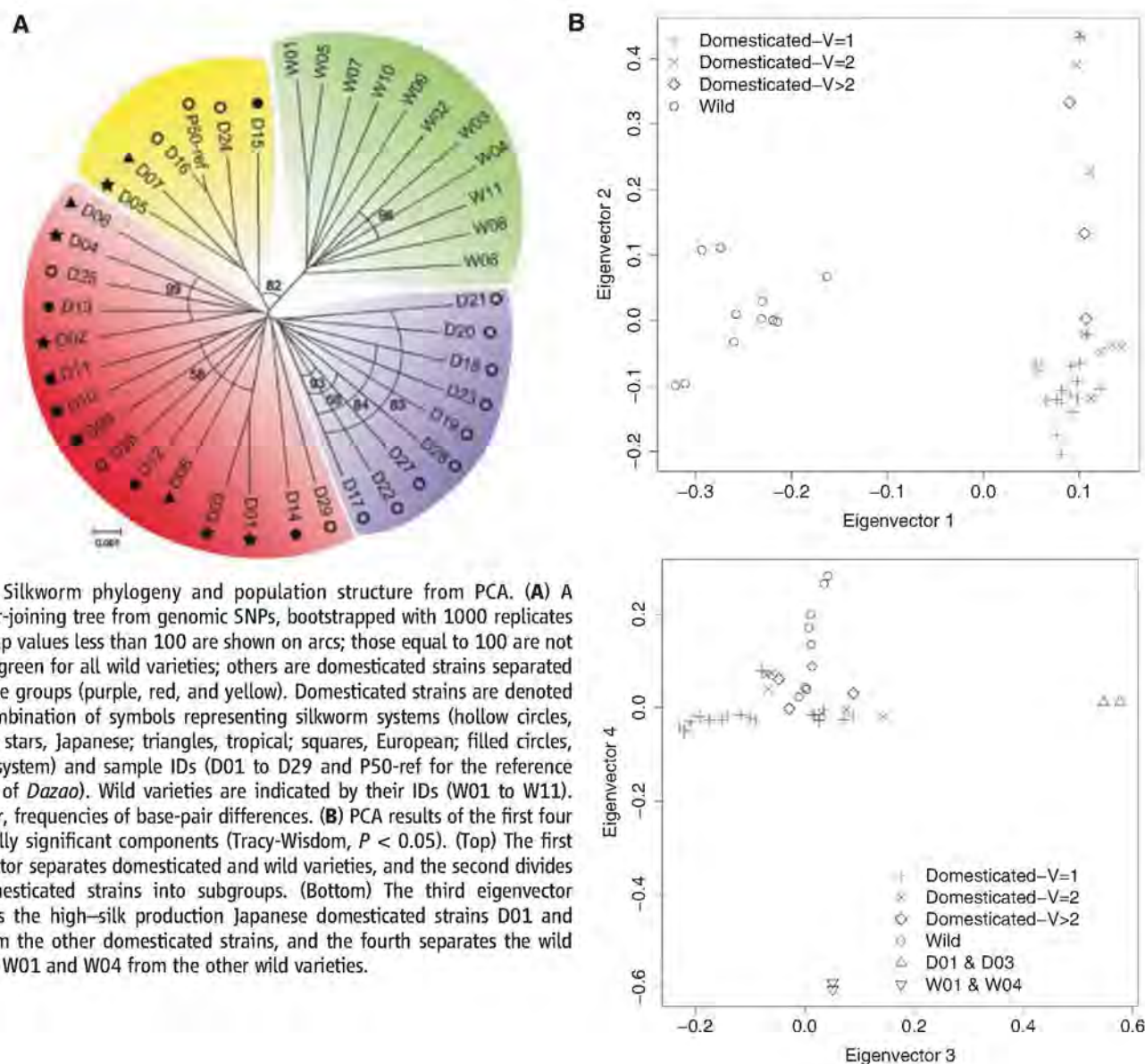


Fig. 1. Silkworm phylogeny and population structure from PCA. (A) A neighbor-joining tree from genomic SNPs, bootstrapped with 1000 replicates (bootstrap values less than 100 are shown on arcs; those equal to 100 are not shown): green for all wild varieties; others are domesticated strains separated into three groups (purple, red, and yellow). Domesticated strains are denoted by a combination of symbols representing silkworm systems (hollow circles, Chinese; stars, Japanese; triangles, tropical; squares, European; filled circles, mutant system) and sample IDs (D01 to D29 and P50-ref for the reference genome of *Dazao*). Wild varieties are indicated by their IDs (W01 to W11). Scale bar, frequencies of base-pair differences. (B) PCA results of the first four statistically significant components (Tracy-Wisdom, $P < 0.05$). (Top) The first eigenvector separates domesticated and wild varieties, and the second divides the domesticated strains into subgroups. (Bottom) The third eigenvector separates the high-silk production Japanese domesticated strains D01 and D03 from the other domesticated strains, and the fourth separates the wild varieties W01 and W04 from the other wild varieties.

tion (I_0) (table S3B). We found that $\theta_{S,domesticated}$ (0.0108) was significantly smaller than $\theta_{S,wild}$ (0.0130) [Mann-Whitney U (MWU), $P = 1.10 \times 10^{-7}$], which may reflect differences in effective population size and demographic history (including domestication and artificial selection). The rate of heterozygosity in domesticated strains was more than twofold lower than that of wild varieties (0.0032 versus 0.0080, respectively) (MWU, $P = 3.33 \times 10^{-6}$). This reduction in heterozygosity is most likely due to inbreeding or the bottleneck experienced by domesticated lines.

In addition to SNPs, we also identified 311,608 small insertion-deletions (indels) (table S4A), a subset of which were validated with polymerase chain reaction (7). The θ_S values for the indels (table S4B) were in agreement with a lower effective population size in domesticated versus wild varieties. A mate-pair relationship method (7, 11) identified 35,093 structural variants (SVs) among the 40 varieties (table S5). Over three-fourths of the SVs overlapped with transposable elements (TEs), suggesting that SV events in silkworm are probably due to TE content (12) and mobility (11). The SNPs, indels, and SVs all con-

tributed to a comprehensive genetic variation map for the silkworm.

To elucidate the phylogeny of silkworms beyond previous studies (6, 13, 14), we used our identified SNPs to estimate a neighbor-joining tree (7) on the basis of a dissimilarity measure of genetic distance (Fig. 1A). This tree represents an average of distances among strains, so lineages cannot be directly interpreted as representing phylogenetic relationships. Instead, the distances may reflect gene flow and other population level processes related to human activities such as ancient commercial trade. The unrooted radial relationship reveals a clear split between the domesticated and wild varieties, and the domestic strains cluster into several subgroups (Fig. 1A).

A principal components analysis (PCA) (7) classified the first four eigenvectors as significant (table S6; Tracy-Widom, $P < 0.05$). The first eigenvector clearly separates the domesticated and wild varieties, whereas the second eigenvector divides the domesticated strains into subgroups correlated with voltinism (Fig. 1B, top). The third principal component separates D01 and D03 (which are high-silk producing Japanese domes-

ticated strains) from the other domesticated strains, whereas the fourth separates W01 and W04 from the other wild varieties (Fig. 1B, bottom). Results of population structure analysis (7) (fig. S3) confirmed the results of the PCA, as well as the neighbor-joining analysis. The clear genetic separation between domesticated and wild varieties suggests a unique domestication event and relatively little subsequent gene flow between the two groups.

One puzzling observation is that, although domesticated strains are clearly genetically differentiated from the wild ones, they still harbor ~83% of the variation observed in the wild varieties. This suggests that the population-size bottleneck at domestication only reduced genetic variability mildly (7); that is, a large number of individuals must have been selected for initial domestication or else domestication occurred simultaneously in many places. To quantify this observation, we fit a simple coalescence-based genetic bottleneck model to the SNP frequency spectrum (7). The estimated model suggests that the domestication event lead to a 90% reduction in effective population size during the initial bottleneck (fig. S2). Additionally, we observed no

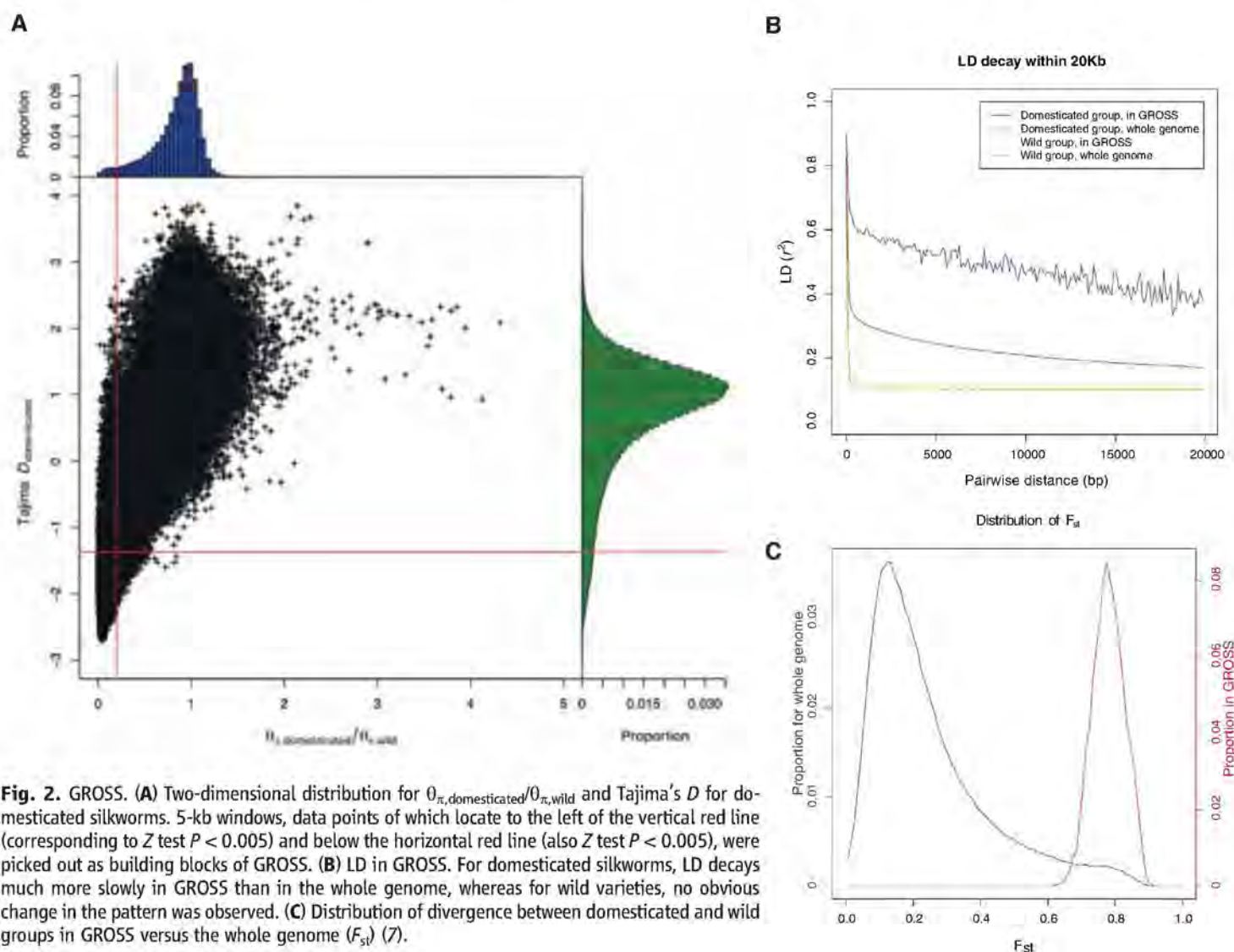


Fig. 2. GROSS. (A) Two-dimensional distribution for $\theta_{S,domesticated}/\theta_{S,wild}$ and Tajima's D for domesticated silkworms. 5-kb windows, data points of which locate to the left of the vertical red line (corresponding to Z test $P < 0.005$) and below the horizontal red line (also Z test $P < 0.005$), were picked out as building blocks of GROSS. (B) LD in GROSS. For domesticated silkworms, LD decays much more slowly in GROSS than in the whole genome, whereas for wild varieties, no obvious change in the pattern was observed. (C) Distribution of divergence between domesticated and wild groups in GROSS versus the whole genome (F_{st}) (7).

excess of low-frequency variants in the domesticated varieties compared with the wild varieties, suggesting that there has not been obvious population growth since the domestication event and that the domestic lines probably have had a generally stable effective population size.

Our measure of pairwise linkage disequilibrium (LD) (7) showed that LD decays rapidly in silkworms, with correlation coefficient r^2 decreasing to half of its maximum value at distances of ~46 and 7 base pairs for the domesticated and wild varieties, respectively (fig. S1). The fast decay of LD implies that regions affected by selective sweeps are probably relatively small. To detect regions with significant (Z test, $P < 0.005$) signatures of selective sweep, we measured SNP variability and frequency spectrum following a genome-wide sliding window strategy (7) (Fig. 2A). Though the significance of our Z tests (7) cannot be interpreted literally due to correlations in LD and shared ancestral history between the two populations, the Z tests suggest differences in frequency spectra and amounts of variability between the two groups. We termed the candidate regions genomic regions of selective signals (GROSS).

We identified a total of 1041 GROSS (7), covering 12.5 Mb (2.9%) of the genome, which may reflect genomic footprints left by artificial selection during domestication. A region affected by selective sweep typically has an elevated level of LD (15, 16), and in our GROSS, the level of LD among SNP pairs less than 20-kb apart was 2.3 times higher than genome average (Fig. 2B), consistent with the hypothesis that selection is affecting these regions. In all these regions, divergence levels (7) between the domesticated and wild groups were also elevated (Fig. 2C), confirming the differentiation of the two subpopulations.

B. mori has experienced intense artificial selection, represents a completely domesticated insect (3), and has become totally dependent on humans for survival. Artificial selection has also enhanced important economic traits such as cocoon size, growth rate, and digestion efficiency (3). Moreover, compared to its wild ancestor *B. mandarina*, *B. mori* has gained some representative behavioral characteristics (such as tolerance to human proximity and handling, as well as extensive crowding) and lost other traits (such as flight, predators, and diseases avoidance). However, to date no genes have been identified as domestication genes under artificial selection. Within GROSS, we identified 354 protein-coding genes that represent good candidates for domestication genes (table S9). Their Gene Ontology annotation (17) showed the most representation in the categories of "binding" and "catalytic" in molecular function, as well as "metabolic" and "cellular" in biological process (fig. S4).

Considering published expression profiles performed on different tissues in fifth-instar day 3 of *Dazao* with genome-wide microarray (18), we found that 159 of our GROSS genes exhibit differential expression. Of these, 4, 32, and 54 genes are enriched in tissues of silk gland, midgut, and

testis, respectively (fig. S5). Among the genes enriched in the silk gland is silk gland factor-1 (*Sgf-1*), a homolog of a *Drosophila melanogaster* *Fkh* gene. *Sgf-1* regulates the transcription of the *B. mori* glue protein-encoding *sericin-1* gene and of three fibroin genes encoding fibroin light chain, fibroin heavy chain, and *fhn/P25* (19, 20). Another silk gland-enriched gene, *BGIBMGA005127*, homologous to the *Drosophila* *sage* gene, was overexpressed fourfold in a high-silk strain compared with *Dazao* (fig. S6). In *Drosophila*, the products of *Fkh* and *sage* genes cooperate to regulate the transcription of the glue genes *SG1* and *SG2*, which are crucial for the synthesis and secretion of glue proteins (21, 22). Additionally, midgut- and testis-enriched genes suggest that genes involved in energy metabolism and reproduction have also been under artificial selection during domestication (7). Specifically, we identified three likely candidates for artificial selection: (i) *NM_001130902* is homologous to paramyosin protein in *Drosophila* and may be related to flight (23). (ii) *NM_001043506* is homologous to fattyacyl desaturase (*desat1*) in *Drosophila*, which is related to courtship behaviors, because mutations in *desat1* can change the pattern of sex pheromones production and discrimination (24). Finally, (iii) *BGIBMGA000972* is homologous to tyrosine-protein kinase *Btk29A* in *Drosophila*, which is involved in male genitalia development (25).

In sericulture, silkworms are typically categorized by their geographic origins (3). Voltinism, which results from adaptation to ecological conditions, and geographic systems have been central to previous studies of silkworm origin and domestication (4–6). Our findings indicate that a unique domestication event occurred and, although voltinism correlates with genetic distances, major genetically cohesive strains cannot be identified on the basis of voltinism. We observed no correlation between longitudes of the sample origins and any of the principal components, but we did find a significant correlation between the latitudes and eigenvectors 2 and 4 in the PCA (table S7). Although this correlation might be due to isolation by distance, this result also agrees with previous studies suggesting that climate affects silkworm biology (2).

The silkworm data reported here represent a large body of genome sequences for a lepidopteran species and offer a source of near-relatives in this clade for comparative genomic analysis. We further proposed a set of candidate domestication genes that, in addition to being putatively under artificial selection, also show higher expression levels in tissues important for silkworm economic traits. Because a proportion of the GROSS genes were probably important in domestication, functional verification of these candidate genes may enable a comprehensive understanding of the differences of biological characteristics between *B. mori* and *B. mandarina*. Moreover, domesticated silkworms have been used as bioreactors (26, 27), and such an effort may provide useful clues to help improve the capacity and capability of silkworms to produce foreign proteins (26). These findings may also aid

in the understanding of how to enhance traits of interest in other organisms in an environmentally safe manner and, because the wild silkworm is a destructive pest, allow new approaches for pest control.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1176620/DC1
Materials and Methods
SOM Text
Figs. S1 to S7
Tables S1 to S10
References

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AMPK Regulates the Circadian Clock by Cryptochrome Phosphorylation and Degradation

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Circadian clocks coordinate behavioral and physiological processes with daily light-dark cycles by driving rhythmic transcription of thousands of genes. Whereas the master clock in the brain is set by light, pacemakers in peripheral organs, such as the liver, are reset by food availability, although the setting, or "entrainment," mechanisms remain mysterious. Studying mouse fibroblasts, we demonstrated that the nutrient-responsive adenosine monophosphate-activated protein kinase (AMPK) phosphorylates and destabilizes the clock component cryptochrome 1 (CRY1). In mouse livers, AMPK activity and nuclear localization were rhythmic and inversely correlated with CRY1 nuclear protein abundance. Stimulation of AMPK destabilized cryptochromes and altered circadian rhythms, and mice in which the AMPK pathway was genetically disrupted showed alterations in peripheral clocks. Thus, phosphorylation by AMPK enables cryptochrome to transduce nutrient signals to circadian clocks in mammalian peripheral organs.

The mammalian hypothalamic suprachiasmatic nucleus (SCN) acts as a master pacemaker, aligning behavioral and physiological rhythms to light-dark cycles (1). Initially, the SCN was thought to be the only site of self-sustaining molecular pacemakers in mammals, but subsequent reports have shown such clocks to be ubiquitous (2, 3). Unlike those in the SCN, clocks in non-light-sensitive organs are entrained by daily feeding (2, 4, 5), which theoretically allows peripheral tissues to anticipate food consumption and to optimize the timing of metabolic processes. Although nuclear receptors and other pathways have been suggested to provide input (6–8), the biochemical basis for non-light-dependent clock entrainment remains unclear.

The adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a central mediator of metabolic signals (9). Nutrient-regulated diurnal phosphorylation of AMPK substrates in rat livers (10) makes AMPK an attractive candidate contributor to peripheral clock entrainment. AMPK is a heterotrimeric protein kinase consisting of a catalytic (α) subunit and two regulatory (β , γ) subunits. Activation of AMPK by liver kinase B1 (LKB1)-mediated (11) or calcium-calmodulin-dependent protein kinase kinase β (CAMKK β)-mediated (12) phosphorylation is increased in the presence of high ratios of AMP to ATP (adenosine triphosphate) or elevated intracellular calcium,

respectively. Biochemical and bioinformatic studies have established the optimal amino acid sequence context in which phosphorylation by AMPK is likely (13, 14), which has facilitated prediction of novel substrates.

Mammalian circadian clocks require alternating actions of activators and repressors of transcription (15). The transcriptional regulators BMAL1 (brain and muscle ARNT-like protein 1) and CLOCK (circadian locomotor output cycles kaput) activate expression of many transcripts including the period (*Per1*, *Per2*, and *Per3*) and cryptochrome (*Cry1* and *Cry2*) genes, whose protein products inhibit CLOCK and BMAL1, which results in rhythmic expression. Posttranslational modification is required for resetting the clock mechanism (15), including ubiquitination of cryptochromes by F-box and leucine-rich repeat protein 3 (FBXL3) (16), which leads to their subsequent degradation.

To explore the role of posttranslational modifications in nutrient-dependent signaling to mammalian peripheral clocks, we used mass spectrometry and bioinformatics to identify likely sites of regulated phosphorylation in mouse CRY1 (fig. S1, A to C). Preliminary biochemical characterization of several observed and predicted sites suggested that modification of serine 71 (S71) and, to a lesser extent, serine 280 (S280) alters the stability of CRY1, and the other mutants examined had less or no effect on stability (fig. S1) (17). Both CRY1 S71 and S280 conform well to the optimal sequence phosphorylated by AMPK (fig. S2). Mutation of either S71 or S280 to a nonphosphorylatable amino acid (alanine) was sufficient to stabilize CRY1, whereas mutation of either S71 or S280 to a phosphomimetic amino acid (aspartic acid) was sufficient to destabilize CRY1 (Fig. 1A). Mutation of S71, which is conserved in all non-light-sensitive insect cryptochromes (6) and higher organisms (fig. S2), to either ala-

nine or to aspartic acid, had a stronger effect than the analogous mutation of S280 (Fig. 1A).

To determine whether the instability of CRY1 phosphomimetic mutants reflects altered interaction with known binding partners, we expressed FLAG-tagged wild-type (WT) or mutant CRY1 with v5-tagged FBXL3 or untagged PER2 and analyzed their interactions by immunoprecipitation of the FLAG-tagged CRY1, followed by immunoblot. Mutation of S71 to aspartic acid (S71D) greatly increased the affinity of CRY1 for FBXL3, in concert with destabilizing CRY1 in the context of FBXL3 overexpression (Fig. 1B and fig. S3A), which suggested that phosphorylation of S71 increases the interaction between CRY1 and FBXL3. CRY1 harboring an S280D mutation coprecipitated double the amount of FBXL3 relative to WT CRY1. In contrast, CRY1-S71D lost the ability to bind PER2, whereas CRY1-S280D retained PER2 binding, as did the other mutants examined (Fig. 1B and fig. S3B). Thus, CRY1-S71D exhibited decreased binding to PER2 and increased binding to FBXL3, each of which is expected to destabilize CRY1 and which together likely account for its instability.

Using purified components, we found that AMPK directly phosphorylates CRY1 in vitro (Fig. 1C). To examine CRY1 S71 phosphorylation in vivo, we tested several phospho-specific antibodies for the ability to recognize WT but not S71A CRY1 phosphorylated by AMPK. We found that an antibody against acetyl coenzyme A carboxylase 1 (ACC1) phosphorylated on S79 indeed specifically recognized CRY1 phosphorylated on S71 (Fig. 1C). To determine whether endogenous AMPK mediates this phosphorylation, we used retroviruses to stably express FLAG-CRY1 in mouse embryonic fibroblasts (MEFs) that were genetically wild-type (WT) or null (*ampka1*^{−/−}; *ampka2*^{−/−}) for the catalytic subunits of AMPK (AMPK^{−/−}). We detected phosphorylation of purified FLAG-CRY1 after treatment with the AMPK agonist aminoimidazole carboxamide ribonucleotide (AICAR) only in the WT cells, which demonstrated that in vivo phosphorylation of S71 is mediated by AMPK (Fig. 1D).

To investigate whether AMPK regulates cryptochrome stability, we generated WT and AMPK^{−/−} cells stably expressing FLAG-tagged WT or doubly nonphosphorylatable (AA) CRY1 and found that WT, but not AA, CRY1 is degraded after treatment with AICAR (Fig. 1E and figs. S3C and S4). The regulation of CRY1 stability by AMPK phosphorylation of S71 and S280 was confirmed by subjecting these cells to a time course of cycloheximide treatment in the presence or absence of AICAR (Fig. 1F and fig. S5). AICAR treatment of WT cells reduced the half-life of WT CRY1 from 45 min to less than 30 min, whereas the half-life of AA CRY1 exceeded 2 hours under all conditions. Genetic loss of AMPK stabilized WT CRY1 but did not affect the stability of the nonphosphorylatable AA mutant, which was more stable than WT CRY1 regardless of the presence or absence of AMPK.

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Fig. 1. Phosphorylation of S71 or S280 by AMPK destabilizes CRY1. **(A)** Human embryonic kidney (HEK) 293 cells expressing WT or mutant FLAG-CRY1 were treated with cycloheximide (CHX) as indicated. AA denotes CRY1 with both S71A and S280A mutations. **(B)** FBXL3-v5 and PER2 bound to FLAG-CRY1 were detected by immunoblot (IB) following immunoprecipitation (IP) from HEK 293 cells. **(C)** (Top) Alignment of the regions surrounding mouse (m) CRY1-S71 and ACC1-S79. (Bottom) In vitro kinase assays using FLAG-CRY1 purified from HEK 293 cells and purified AMPK (6). Phosphorylated CRY1 was detected by radiography (left) or IB using an antibody against phosphoACC1-S79 (right). **(D)** Phosphorylation of stably expressed FLAG-CRY1 by endogenous AMPK was detected by IB following IP from MG132-treated MEFs. **(E)** MEFs were treated with vehicle (–) or 2 mM AICAR (+) for 2 hours. **(F)** MEFs were treated with CHX ± 2 mM AICAR as indicated. CRY1 was detected by IP and IB for the FLAG epitope in (C to F). Graphs in (F) represent the means ± SD of three quantified IB samples per condition.

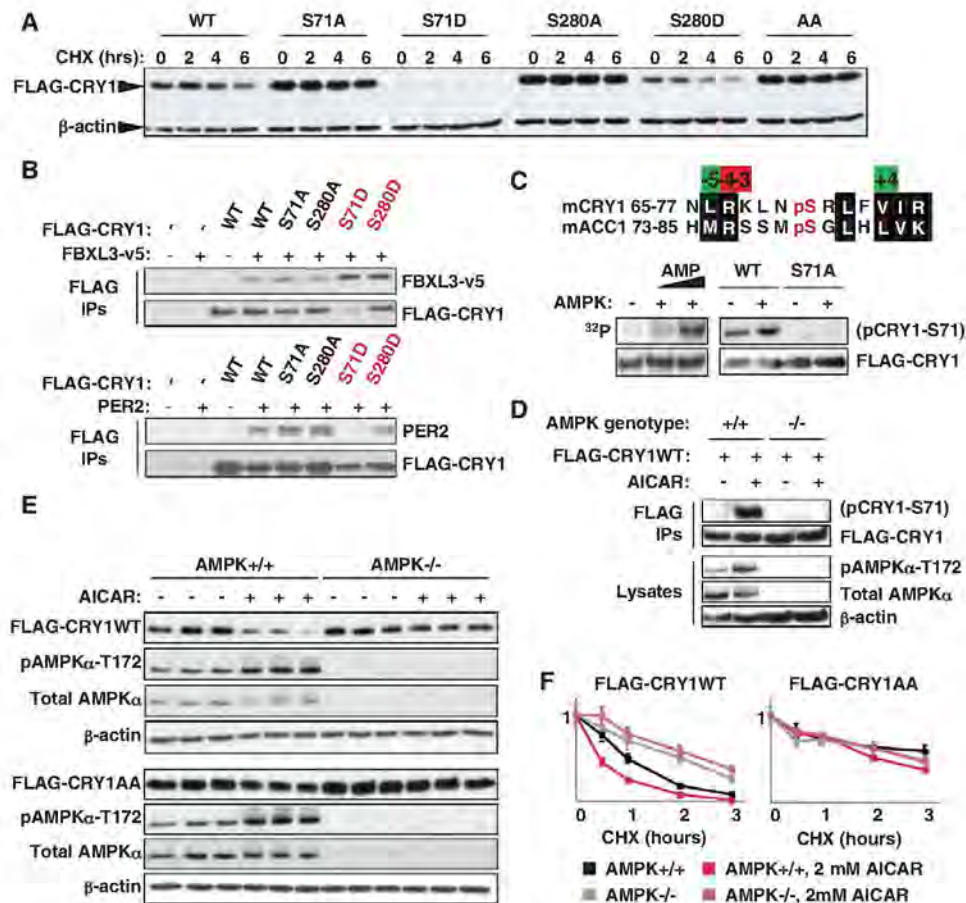
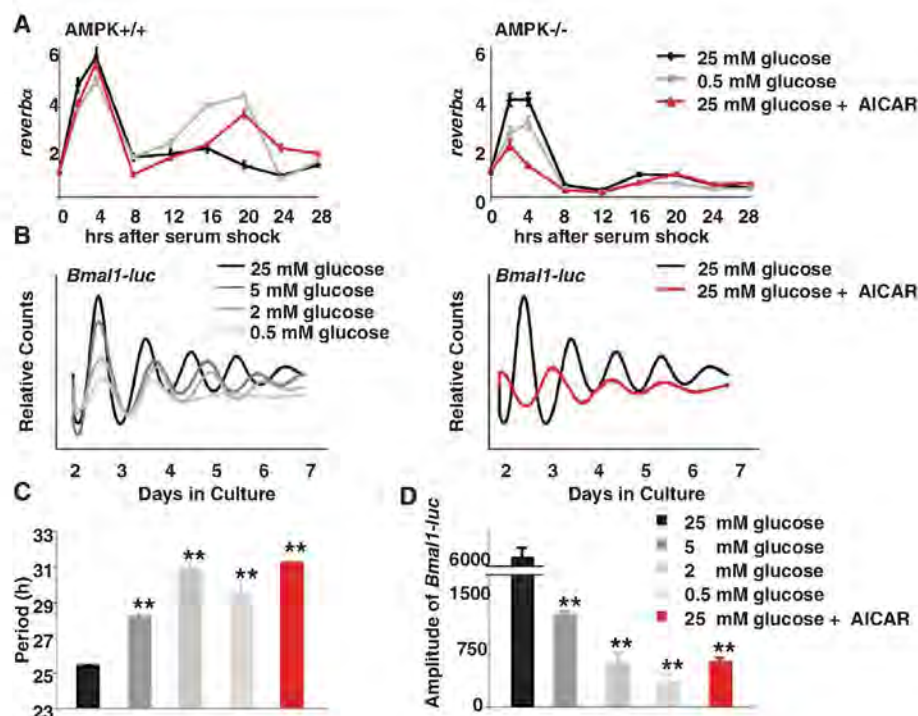


Fig. 2. Disruption of AMPK signaling alters circadian rhythms in MEFs. **(A)** MEFs were synchronized by serum shock and transferred to media containing glucose and AICAR as indicated. Quantitative PCR (QPCR) was performed by using cDNA from samples collected at the indicated times. Data are means ± SD of a representative experiment analyzed in triplicate. **(B)** Typical results of continuous monitoring of luciferase activity from U2OS cells stably expressing *Bmal1-luciferase*. **(C)** and **(D)** Quantification of the circadian period (C) and amplitude (D) of *Bmal1*-driven luciferase from experiments performed as described in (B). Data in (C) and (D) are means ± SD for four samples per condition. Analysis of variance (ANOVA) indicated a significant difference between categories. ***P* < 0.01 versus samples cultured in 25 mM glucose.



Given the importance of feeding-derived signals for circadian clock resetting, the regulation of AMPK by glucose availability, and the destabilization of cryptochromes by AMPK, we examined the effects of AMPK expression and glucose availability on circadian rhythmicity in fibroblasts (6). When WT fibroblasts were cul-

tured in medium containing either limiting glucose or AICAR, the amplitude of circadian expression of *REV-ERBα* (*reverba*, also known as nuclear receptor subfamily 1, group D, member 1, *Nr1d1*) and D-site albumin-binding protein (*dbp*) was enhanced (Fig. 2A and fig. S6), consistent with a model in which glucose deprivation ac-

tivates AMPK and reduces CRY1 stability, leading to de-repression of *CLOCK:BMAL1* targets. Notably, neither glucose deprivation nor AICAR affected the expression of *reverba* and *dbp* in MEFs lacking either *LKB1* or AMPK (Fig. 2A and fig. S6, B to D), which indicates that these effects of glucose limitation are mediated by AMPK, al-

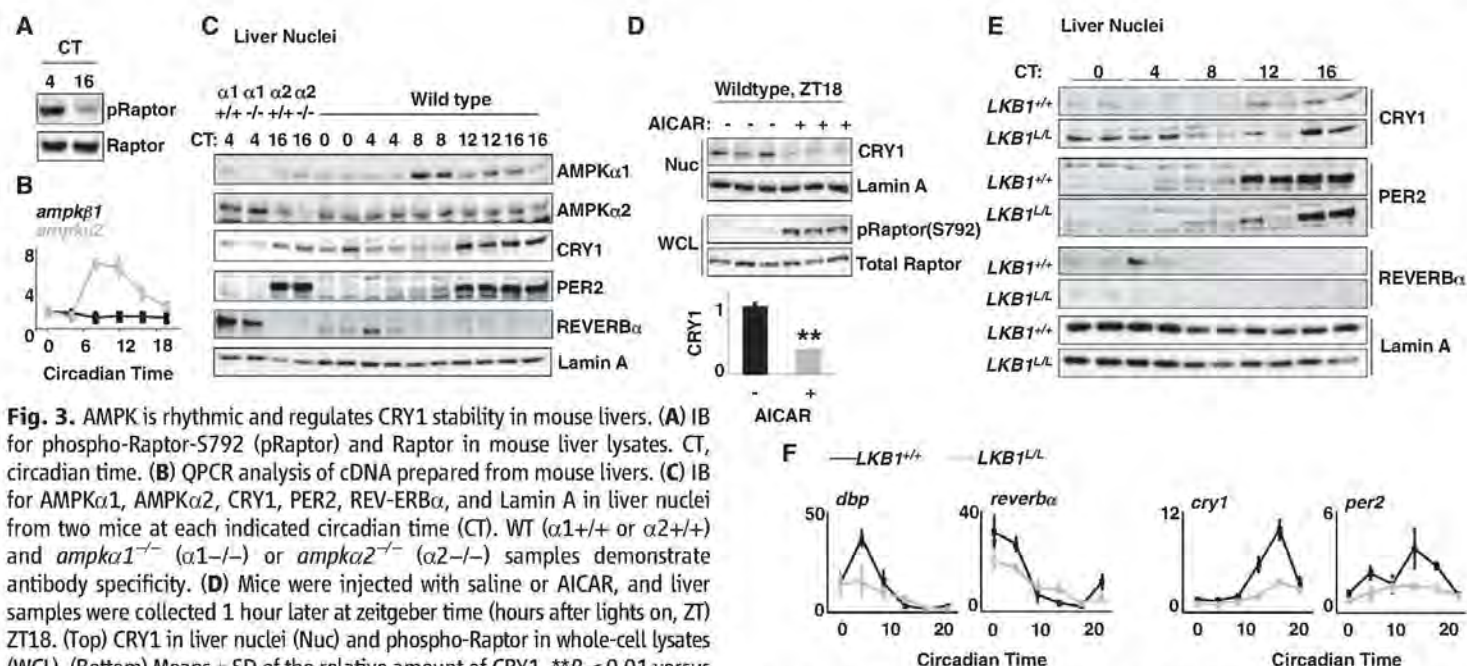


Fig. 3. AMPK is rhythmic and regulates CRY1 stability in mouse livers. (A) IB for phospho-Raptor-S792 (pRaptor) and Raptor in mouse liver lysates. CT, circadian time. (B) QPCR analysis of cDNA prepared from mouse livers. (C) IB for AMPK α 1, AMPK α 2, CRY1, PER2, REV-ERB α , and Lamin A in liver nuclei from two mice at each indicated circadian time (CT). WT (α 1 $^{+/+}$ or α 2 $^{+/+}$) and *ampk* α 1 $^{-/-}$ (α 1 $^{-/-}$) or *ampk* α 2 $^{-/-}$ (α 2 $^{-/-}$) samples demonstrate antibody specificity. (D) Mice were injected with saline or AICAR, and liver samples were collected 1 hour later at zeitgeber time (hours after lights on, ZT) ZT18. (Top) CRY1 in liver nuclei (Nuc) and phospho-Raptor in whole-cell lysates (WCL). (Bottom) Means $\pm$ SD of the relative amount of CRY1. ** P < 0.01 versus saline-treated samples. (E) CRY1, PER2, REV-ERB α , and Lamin A detected by IB in liver nuclei from *LKB1* $^{+/+}$ and *LKB1* $^{-/-}$ mice. (F) QPCR for *dbp*, *reverba*, *cry1*, and *per2* in mouse liver. All transcripts were normalized to *u36b4*.

though other glucose-dependent signaling pathways may also affect circadian clocks (18).

The *Bmal1* promoter is repressed by REV-ERB α . Therefore, we examined the effects of reducing glucose availability on circadian rhythms using fibroblasts stably expressing luciferase under the control of a *Bmal1* promoter. In a standard (high-glucose) medium, *Bmal1-luciferase* exhibited a high-amplitude circadian rhythm with a period of 25.3 hours (Fig. 2, B and C). Decreasing the amount of glucose in the medium increased the circadian period up to 30.7 hours and decreased the amplitude of *Bmal1-luciferase* expression (Fig. 2D). AICAR treatment had similar effects on circadian period and amplitude (Fig. 2, B to D), which reinforced the idea that the circadian effects of glucose limitation are mediated by AMPK. We also found evidence to suggest that AMPK activation shifts the phase of entrainment in mouse fibroblasts and mouse livers (6) (figs. S7 and S8).

If AMPK-directed cryptochrome phosphorylation regulates the phase of peripheral clocks, the activity, expression, and/or localization of AMPK must be subject to diurnal regulation. Studying mouse liver, we found that the phosphorylation of AMPK substrates Raptor-Ser792 and ACC1-Ser79 was reproducibly higher during the subjective day than at night (Fig. 3A and fig. S9). We also observed circadian expression of the mRNA encoding the regulatory AMPK β 2 subunit (Fig. 3B and fig. S10). Because the subunit composition of AMPK complexes regulates its localization (19), oscillating AMPK β 2 could diurnally regulate the nuclear import of AMPK. Indeed, AMPK α 1 exhibited a robust circadian rhythm of nuclear localization (Fig. 3C and fig. S11A), peaking synchronously with *ampk* β 2 expression. The time of peak AMPK α 1 nuclear localization

coincides with minimum nuclear CRY1 (Fig. 3C), which suggests that rhythmic nuclear import of AMPK may contribute to the AMPK-mediated phosphorylation and degradation of cryptochromes.

To determine whether AMPK affects circadian clocks in vivo, we measured CRY1 protein in liver nuclei from mice injected with either saline or AICAR during the early nighttime hours. AMPK activation reduced endogenous CRY1 by an average of 67% in vivo (Fig. 3D). To specifically examine AMPK's role in the liver, we studied circadian proteins and transcripts in the livers of control mice (*LKB1* $^{+/+}$) or littermates harboring loss of *lkb1* in hepatocytes (*LKB1* $^{-/-}$). Liver-specific deletion of *lkb1* abolished AMPK activation in that organ (20) and significantly increased the amount of CRY1 present in liver nuclei across the circadian cycle, particularly during the daytime hours when AMPK was most active in control mice (Fig. 3E and fig. S11B). This increase was associated with decreased REV-ERB α and decreased amplitude of circadian transcripts throughout the circadian cycle (Fig. 3F). Thus, loss of AMPK signaling in vivo stabilizes cryptochromes and disrupts circadian rhythms, consistent with the hypothesis that this pathway contributes to the metabolic control of light-independent peripheral circadian clocks.

In summary, we provide evidence that mammalian cryptochromes, which evolved from blue light photoreceptors, have been repurposed by AMPK to act as chemical energy sensors and can transduce nutrient signals to clocks (fig. S12). Although our data suggest that AMPK phosphorylation of cryptochromes contributes to metabolic entrainment of peripheral clocks, we expect that the communication of nutritional status to clocks is complex and that additional pathways contribute in vivo (6). Given that AMPK is a

central regulator of metabolic processes, the rhythmic regulation of AMPK has implications for the circadian regulation of metabolism. Genetic alteration of circadian clocks either ubiquitously (21, 22) or in a tissue-specific manner (23, 24) elicits dramatic changes in feeding behavior, body weight, running endurance, and glucose homeostasis, each of which is also altered by manipulation of AMPK (25–30). The abilities of AMPK to mediate circadian regulation and of CRY1 to function as a chemical energy sensor suggest a close relation between metabolic and circadian rhythms.

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Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S9

Tables S1 to S3

References

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Teachers' Participation in Research Programs Improves Their Students' Achievement in Science

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Research experience programs engage teachers in the hands-on practice of science. Program advocates assert that program participation enhances teachers' skills in communicating science to students. We measured the impact of New York City public high-school science teachers' participation in Columbia University's Summer Research Program on their students' academic performance in science. In the year before program entry, students of participating and nonparticipating teachers passed a New York State Regents science examination at the same rate. In years three and four after program entry, participating teachers' students passed Regents science exams at a rate that was 10.1% higher ($P = 0.049$) than that of nonparticipating teachers' students. Other program benefits include decreased teacher attrition from classroom teaching and school cost savings of U.S. \$1.14 per \$1 invested in the program.

U.S. high-school students perform less well in science, technology, engineering, and math (STEM) than students in other economically advanced countries do (1–5). This low performance of students in STEM and the relative paucity of U.S. students pursuing STEM careers led one of the authors (S.C.S.) to found Columbia University's Summer Research Program (CUSRP) (6). Other universities have implemented similar programs for STEM teachers (7), likely for similar reasons.

CUSRP's premise, like that of other research experience for teachers programs, is that experience in the practice of science improves the quality and authenticity of science teaching and thereby increases student interest and achievement in science. CUSRP differs from most STEM summer programs in the time that participants are engaged [16 weeks over two summers, supporting online material (SOM) text S1], in the intensity of professional development (1 day per week, SOM text S2), and in its focus on quantitative assessment of the impacts of teacher participation on student science achievement.

Each spring, the CUSRP's Advisory Committee selects 10 to 13 middle- and high-school science

teachers [~90% from New York City (NYC) public middle and high schools] from a pool of 30 to 60 applicants. (See SOM text S3 for teacher selection criteria, table S1 for program costs, and tables S2 to S4 for demographics of applicants and participants.) Admitted teachers are appointed as Visiting Scholars at Columbia University. They receive a stipend of \$6000 per summer and an e-mail account enabling them to use the university's libraries. To aid in the transfer of concepts and techniques learned at Columbia to their schools and students, teachers may request up to \$1000 per year for classroom and/or lab supplies and equipment and for conference travel. Per university regulations (8), entering teachers are trained in laboratory safety and other areas as needed (SOM text S4).

Accepted teachers are referred to a Columbia University faculty member who has indicated a willingness to mentor a teacher and is working in an area of interest to the teacher. Both the teacher and his/her prospective faculty mentor must indicate their agreement to work with one another for CUSRP to confirm the placement (see SOM text S5). Each host laboratory receives \$1000 per summer for teacher-related costs (table S1). With his/her faculty mentor's approval, a graduate student with whom a teacher worked during the summer may be paid by CUSRP for consulting with the teacher and for providing him/her with in-school assistance (SOM text S6). Total program costs for the two-summer program are \$27,526 per teacher (table S1), which are funded by grants and gifts (6).

Teachers assemble for 1 day each week during the summer for professional development exer-

cises, including seminars, science museum visits, demonstrations of science teaching and teaching materials, training in data-driven instruction and classroom transfer of science concepts and technologies, and teacher-led research presentations. CUSRP provides lunch on professional development days, thereby encouraging social and professional interactions that facilitate the coalescence of teachers into a professional learning community (9). By these means, CUSRP has developed academically prepared, experienced teachers with the laboratory and science experiment-management skills needed to affect students' science achievement.

Of the 145 teachers who completed the program from 1994 to 2005, 95 taught a Regents-level science course in a regular NYC public high school and therefore were eligible for the student outcomes study (table S5). The number of teachers available to participate in the study was reduced to 32 because of reassignments to teach a non-Regents subject, transfer to another school, lack of a nonparticipating teacher concurrently teaching the same subject, or a school-wide Regents exam waiver (SOM text S7 and tables S5 and S6). These 32 teachers were similar in race, gender, age, educational background, and teaching experience to the 145 teachers who completed CUSRP from 1994 to 2005 and the 95 who taught in NYC public high schools (tables S2 to S5).

From 1998 to 2002, CUSRP participated in a National Science Foundation-sponsored multi-site study of science work experience programs for teachers (SWEPT), which encompassed teachers and students in more than 80 urban and suburban high schools in Arkansas, California, Georgia, Idaho, New York, and Washington. The study's results generalize to CUSRP (10). Thus, it is not surprising that we found rates of and reasons for teacher attrition similar to those reported by the SWEPT study (SOM text S7).

To assess program impact on teachers, CUSRP teachers were surveyed in the spring of their first and second years after program entry about their current professional activities. Their responses (Table 1) indicate that they implemented a number of new constructivist educational practices. A trained observer (11) repeatedly visited classes of one cohort of program participants and confirmed the accuracy of these self-reports (Table 1). Consistent with this observer's findings and our comment that the SWEPT study's (10) results generalize to CUSRP, a significantly ($P < 0.05$) higher percent-

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age of SWEPT teachers' students reported reflecting "on course material by writing in a notebook," using "primary sources such as journals," and being encouraged by their teacher to "think about a math/science career" than comparison teachers' students did (table S8) (10).

CUSRP teachers indicated that the stresses they experienced in adapting to a research laboratory increased their appreciation of their students' difficulties and prompted them to respond more sympathetically to them. Instead of judging students' answers as "right" or "wrong," they ask, "Why do you think that?" They gain the confidence to acknowledge gaps in their own knowledge. "That's a good question," they say. "I don't know the answer but I can help you find it."

As in other research experience programs (12), CUSRP participants reported that their experiences in the program elevated their interest in education and encouraged them to continue teaching. Accordingly, attrition rates were 2.3% per year for CUSRP graduates versus 6.3% per year for comparably experienced teachers nationally (SOM text S7 and tables S6 and S9).

To earn a New York State (NYS)-certified high-school diploma, students must score $\geq 65\%$ on five NYS Regents exams, one of which must be in science. Hence, Regents are high-stakes exams on which students strive to do their best. We compared pass rates on Regents biology (termed "Living Environment" in NYS), chemistry, and earth science exams of 7209 students of CUSRP teachers with those of 36,101 students who studied the same subjects at the same time and in the same schools in classes of non-CUSRP teachers as a quantitative measure of the impact of teacher participation in CUSRP on student achievement (Fig. 1 and table S10).

In the year before CUSRP entry, 45.7% of CUSRP teachers' students passed ($\geq 65\%$) a Regents science exam, essentially the same percentage as students of nonparticipating teachers (Fig. 1). In the first 2 years after teacher entry into CUSRP, their students' Regents science exam pass rates were not significantly different from those of nonparticipating teachers' students. In contrast, in the third and fourth years after teacher entry into CUSRP, their students' average Regents science exam pass rates were 10.1 percentage points higher than those of nonparticipating teachers' students (Fig. 1). This difference was significant ($P = 0.049$) by standard one-way analysis of variance (13) using SPSS software.

Overall, in the 4 years after each teacher's entry into CUSRP, an average of 15.5 more of her/his students passed a Regents science exam than nonparticipating teachers' students did (table S10, row 9). This stepwise improvement in Regents exam pass rate is the pattern that is expected after teacher adoption of new teaching methods and materials. It is consistent with Supovitz's report (14) that teachers take several years to translate professional development experiences into new educational practices.

Three lines of evidence indicate that these results were not influenced by systematic bias.

First, pre-course cognitive tests and surveys administered to 4397 high-school freshmen and sophomores in biology and chemistry classes of study and comparison teachers participating in the national SWEPT study showed no significant differences in cognitive abilities, home educational resources, parental education, or student or parental educational expectations among the CUSRP teachers' students and those of nonparticipating teachers (10). Four hundred and seventy of these students were enrolled in Regents living environment or chemistry classes of 12 CUSRP and 12 comparison teachers in NYC public high schools. The sole significant ($P < 0.04$) difference among the NYC students was that more CUSRP than comparison teachers' students reported that their parents expected them to attend college. Thus, schools did not assign demographically or academically advantaged students to CUSRP teachers' classes.

Second, teachers whose students' Regents exam results make up the substantive data set (Fig. 1) taught at 24 different regular public high schools in all five NYC boroughs. The socioeconomic characteristics of students in these schools mirror those of NYC's public schools generally [71% are minorities underrepresented in science and 75% qualify for free lunch (15, 16)]. That they were from disadvantaged backgrounds is manifest in their Regents science exam pass rates (Fig. 1), 11.5 to 13.4 percentage points less than

the NYC-wide average for living environment, chemistry, and earth science (table S11).

Third, the study's design controlled for the most important variables [for example, teachers' and students' socioeconomic backgrounds, students' cognitive abilities, school environment, and curriculum (10)]. The use of Regents exams assured that students took tests seriously (10). Teachers' full-time, two-summer-long engagement precluded their participation in another sustained professional development activity in years one and two after entry into CUSRP.

Thus, teacher motivation remains the principal uncontrolled variable. Ideally, one would control for it by randomly assigning teachers to control and experimental groups. This would require two applicants of equivalent background and experience teaching the same subject in the same or similar high school per program opening. NYC's public school system is the nation's largest. Nonetheless, the number of qualified applicants was not sufficient to yield 20 applicants from the same or similar high schools annually. Therefore, random assignment was not an option.

Teachers who volunteer for two summers of immersion in research are probably highly motivated, increasing the likelihood that they will outperform their nonparticipating colleagues. Yet, in the year preceding CUSRP entry, we found no significant difference in Regents science exam performance of participating and nonpar-

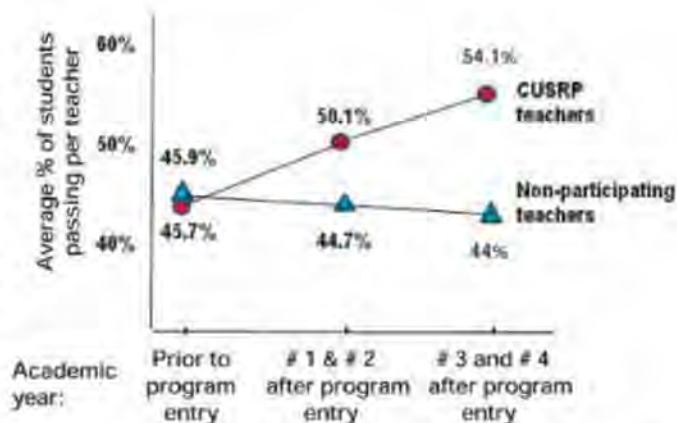
Table 1. Responses to CUSRP's Spring Implementation Survey (table S7) of 46 (90%) of 51 teachers who participated in, or completed, the program in 2004 and 2005.

Responses

- 96% increased hands-on classroom activities and/or introduced new laboratory exercises.
- 93% developed new or revised content to lessons and/or laboratories.*
- 83% introduced new technologies in their class and laboratory exercises (e.g., chromatography, micro-pipettes, PowerPoint).
- 73% increased requirements for formal written and/or oral reports.
- 65% read scientific journals (e.g., *Science*, *Nature*) more frequently.
- 64% discussed science careers and related jobs with their students.
- 53% assumed new leadership roles/responsibilities in their schools or districts.

*361 standards-based lesson plans are currently on CUSRP's Web site (6).

Fig. 1. Percent of CUSRP teachers' and nonparticipating teachers' students passing Regents examinations. Data are for 7209 students in living environment, chemistry, or earth science classes of NYC public high-school science teachers participating in CUSRP (red circles) and for 36,101 students studying the same subject at the same time and in the same high-school in a class taught by a nonparticipating science teacher (blue triangles) (see tables S2 to S5 for demographics of teachers and table S10 for the percent and number of additional students passing a Regents science examination per CUSRP teacher versus per nonparticipating teacher).



ticipating teachers' students (Fig. 1). This finding suggests that although teacher motivation probably contributes to their students' improved achievement, it does not predestine this outcome. The evidence suggests that the program's impact on teachers drives the improvements in student science achievement (Fig. 1 and table S10).

What elements account for the program's success? Clearly, the laboratory experience is one. In the sense that teachers work in different laboratories with different mentors on different research problems, each teacher's experiences are unique. What is common is that all teachers are treated as professionals, challenged to think independently and creatively, and engaged in studying an authentic contemporary scientific problem. This stretches teachers professionally and personally, sharpens their analytic and laboratory experiment-management skills, and enables them to better understand and communicate science concepts and practices.

The weekly day-long professional development workshops and the two-summer requirement also are critical for the program's success (SOM text S1 and S2). The workshops are weekly reminders that the program's purpose is to enhance teachers' abilities as science educators. The two-summer commitment ensures that teachers achieve competence and confidence in their lab experiment-management skills, which are attributes that characterize teachers whose students performed at the highest level on the *National Assessment of Educational Progress* 1996 (17) and 2005 (18) 8th-grade science tests. The authors of these studies concluded that such teachers are more likely to implement weekly science demonstrations and hands-on exercises than are teachers lacking these skills, and students who participate in such exercises weekly exhibit greater science achievement than students who participate in them less frequently or not at all. We suggest that the skills teachers acquire in science research programs are qualitatively distinct from, and complementary to, those acquired in most pre-service academic training. They encourage teachers to implement authentic science exercises that engage students' interest and stimulate them to inquire about the natural world. As one CUSRP teacher put it, "Before I entered this program, I taught about chemistry. Now I teach chemistry."

The increases in student science achievement and teacher retention provide immediate economic benefits to NYC's Department of Education (DOE) (tables S12 and S13) and longer-term economic benefits to society as a whole (19). NYC's high schools require students who fail a Regents exam to retake and pass it. At \$2107 per student per course, we estimate that NYC's DOE saved \$297,845 in summer school, course, and exam repetition costs for the 155 additional students who passed a Regents science exam of each cohort of 10 CUSRP teachers (table S12, row 2). CUSRP teachers are retained in education at a ~threefold greater rate than comparably experienced science teachers are (SOM text S7 and table S9). We estimate that in the 4 years after CUSRP entry of each cohort of 10 teachers,

NYC's DOE saved \$17,055 in teacher recruitment costs (table S12, row 3). Overall, we estimate that NYC's DOE saved \$1.14 per \$1 invested by CUSRP's sponsors (table S12, row 5).

Biology (Living Environment, chemistry, and earth science Regents exams are collectively the second-least-frequently passed Regents exams among NYC high-school students (table S11). If 10% of the 15.5 additional students of each CUSRP teacher who passed a Regents exam also earn a Regents high-school diploma, each CUSRP teacher will have produced 1.55 additional Regents high-school diplomates in the 4 years after program entry (table S12, row 6). The public economic benefits of high-school graduates exceed those of nongraduates by \$209,100 (19). Thus, 1.55 additional high-school graduates generate tax revenues and societal cost savings of \$282,841 (table S13, row 7), a return of \$10.27 per \$1 invested in CUSRP (table S12, row 8). CUSRP's overall return rate, therefore, probably falls between the \$1.14 in immediate economic benefits and \$10.27 in long-term economic benefits per \$1.00 invested.

More than 28,500 students studied Regents-level Living Environment, chemistry, or earth science in classes of the 95 NYC public high-school science teachers (table S13) who completed CUSRP between 1994 and 2005. We estimate that during this period, 2339 (8.25%) (table S13) more CUSRP than non-CUSRP teachers' students passed a Regents exam in one of these sciences and that 15 more CUSRP than non-CUSRP teachers (table S13) were retained in classroom teaching. Accordingly, NYC's DOE saved \$5.3 million in course repetition and teacher recruitment costs (table S13), or \$2.01 per \$1.00 that CUSRP's sponsors invested in the program. If in addition, 10% (234) of these students earned Regents diplomas, the United States will benefit from the ~\$42.8 million (calculated as in table S12, row 7) in additional lifetime tax payments and health, welfare, and criminal justice cost savings they generate. CUSRP's ease of implementation, effectiveness, and individual and societal benefits have led to its adoption by Singapore's Ministry of Education (20). We suggest that it is a model for in-service science teacher professional development that significantly improves student science achievement.

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Supporting Online Material

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References and Notes

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The Taste of Carbonation

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Carbonated beverages are commonly available and immensely popular, but little is known about the cellular and molecular mechanisms underlying the perception of carbonation in the mouth. In mammals, carbonation elicits both somatosensory and chemosensory responses, including activation of taste neurons. We have identified the cellular and molecular substrates for the taste of carbonation. By targeted genetic ablation and the silencing of synapses in defined populations of taste receptor cells, we demonstrated that the sour-sensing cells act as the taste sensors for carbonation, and showed that carbonic anhydrase 4, a glycosylphosphatidylinositol-anchored enzyme, functions as the principal CO₂ taste sensor. Together, these studies reveal the basis of the taste of carbonation as well as the contribution of taste cells in the orosensory response to CO₂.

Humans perceive five qualitatively distinct taste qualities: bitter, sweet, salty, sour, and umami (a savory sensation characterized by the taste of monosodium glutamate). Sweet and umami are sensed by members of the T1R family of heterotrimeric guanine nucleotide binding protein (G protein)-coupled receptors (GPCRs) (1–3); bitter stimuli are detected by

T2R GPCRs (4–7); and sourness is sensed by cells expressing the ion channel PKD2L1 (8–10). In the tongue, these receptors function in distinct classes of taste cells, each tuned to a specific modality (7, 8, 11, 12).

In addition to these well-known stimuli, the taste system appears to be responsive to CO₂ (13–15). Mammals have multiple sensory systems that respond to CO₂, including nociception (16, 17), olfaction (18), and chemoreception essential for respiratory regulation (19), yet the molecular mechanisms for CO₂ reception remain poorly defined. Thus, we wondered how taste receptor cells (TRCs) detect and respond to carbonation.

We studied the electrophysiological responses of TRCs to CO₂ by recording tastant-induced action potentials from one of the major nerves innervating TRCs of the tongue [chorda tympani (15)]; this physiological assay monitors the activity of the gustatory system at the periphery and provides a reliable measure of TRC func-

tion (12, 20). Indeed, the taste system displayed robust, dose-dependent, and saturable responses to CO₂ stimulation. The responses were evident for carbonated drinks (e.g., club soda), CO₂ dissolved in buffer, and even direct stimulation of the tongue with gaseous CO₂ (Fig. 1). In contrast, stimulation with pressured air did not elicit any gustatory response (Fig. 1).

To define the identity of the TRCs needed to taste carbonation, we examined CO₂ responses from engineered mice in which specific populations of TRCs were genetically ablated by targeted expression of attenuated diphtheria toxin [e.g., sweetless, umamiless, sourless mice, etc. (8, 21)] and determined whether their taste systems remained responsive to CO₂. Selective ablation of sour sensing (i.e., PKD2L1-expressing) cells not only abolished all gustatory responses to acidic stimuli, but also eliminated responses to gaseous or dissolved CO₂ (Fig. 1 and fig. S1). These results show that PKD2L1-expressing cells are essential for CO₂ detection.

To identify a candidate CO₂ receptor, we carried out gene expression profiling of sour cells. We reasoned that transcripts for genes involved in carbonation sensing should be enriched in PKD2L1-expressing cells, but that such transcripts would be relatively rare in taste tissue in which PKD2L1 cells have been ablated. Thus, we conducted complementary microarray experiments using mRNA isolated from hand-picked green fluorescent protein (GFP)-labeled sour TRCs, and as a counterscreen, with mRNA from taste buds of animals lacking sour-sensing cells [PKD2L1-DTA mice (8)]. One gene, *Car4*, was particularly attractive: It was highly specific for PKD2L1-expressing cells versus other TRC types

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Fig. 1. PKD2L1-expressing sour-sensing cells mediate taste responses to carbonation. **(A)** Wild-type mice show neural responses to carbonated solutions and carbon dioxide. Shown are chorda tympani nerve responses to control sweet (30 mM acesulfame K, AceK), bitter (10 mM quinine, QUI), salty (120 mM NaCl), amino acid (30 mM monopotassium glutamate + 0.5 mM inosine monophosphate, MPG), and sour (50 mM citric acid) stimuli as well as carbonated water (club soda) and gaseous CO₂ normalized to the responses to 250 mM NaCl (NR; see supporting online text). **(B)** Dose response to CO₂ in wild-type mice or in animals lacking sour-sensing cells (PKD2L1-DTA) and in control animals lacking sweet-sensing cells (T1R2-DTA). **(C)** Quantitation of carbon dioxide responses in wild-type ($n = 6$), T1R2-DTA ($n = 4$), and PKD2L1-DTA ($n = 5$) animals. Values are means $\pm$ SEM of normalized chorda tympani responses.

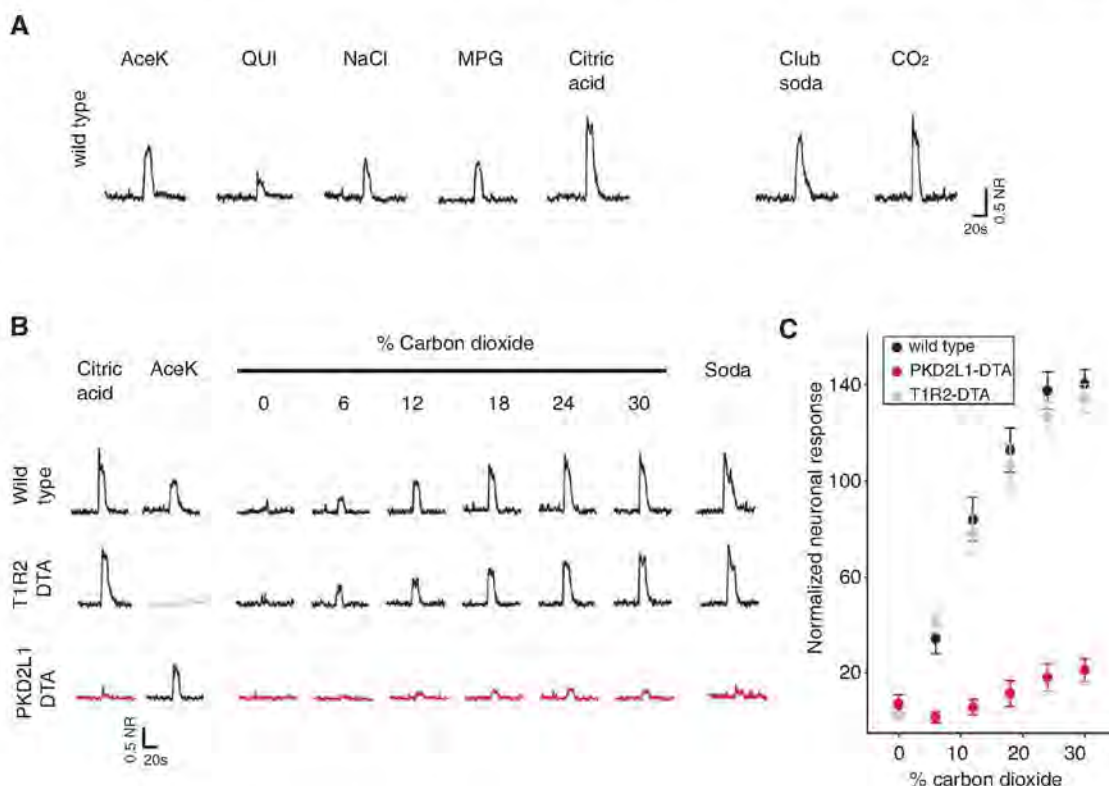


Fig. 2. Selective localization of carbonic anhydrase 4 to PKD2L1-expressing sour cells. (A) Immunohistochemical staining of Car4 expression (lower panel, red) in taste buds of transgenic mice in which sour-sensing cells were marked by GFP fluorescence (PKD2L1-GFP; upper panel, green); large panel shows the superimposed double labeling. (B) Diphtheria toxin-mediated ablation of sour cells. Upper panel: Double-label immunofluorescence with a marker of TRCs, claudin 7 (Cldn7, blue), and antibodies to Car4 (red). Lower panel: Labeling in PKD2L1-DTA mice stained as above. Shown are sections of foliate papillae; equivalent results were obtained in taste buds from other regions of the oral cavity (fig. S4).

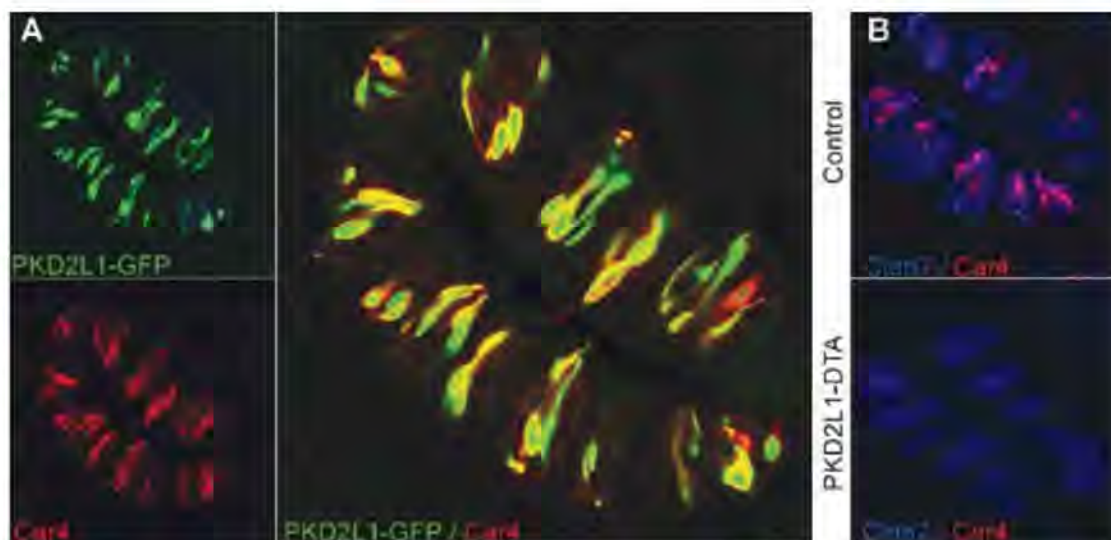
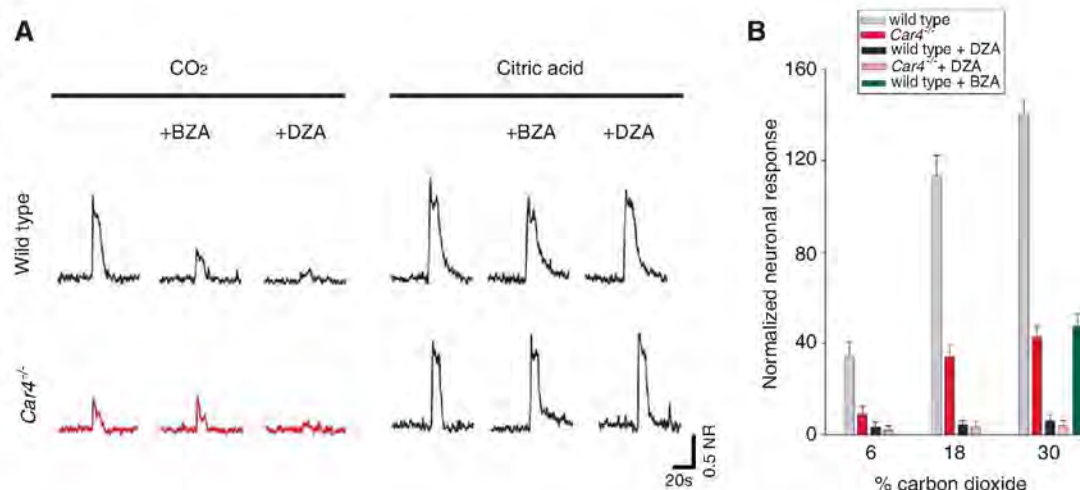


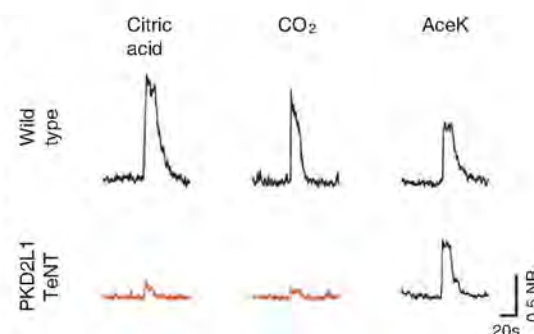
Fig. 3. Requirement of carbonic anhydrase 4 for taste responses to carbon dioxide. (A) Representative integrated chorda tympani responses to CO₂ and sour stimulation in wild-type or *Car4*^{-/-} animals exposed to the cell-impermeant carbonic anhydrase inhibitor benzolamide (BZA) or the cell-permeant, broad-spectrum inhibitor dorzolamide (DZA). (B) Quantitation of carbon dioxide responses in wild-type and *Car4*^{-/-} animals; means $\pm$ SEM ($n = 6$). Green bar denotes wild-type responses to 30% CO₂ in the presence of BZA. See fig. S5 for responses to other taste stimuli.



(Fig. 2), and moreover it encodes carbonic anhydrase 4, a member of a large family of enzymes implicated in sensing, acting on, and responding to CO₂ in various systems, including chemosensation (17–19, 22–28).

Carbonic anhydrases (CAs) reversibly catalyze the conversion of CO₂ into bicarbonate ions and free protons (29, 30). Car4 is a mammalian carbonic anhydrase that functions as an extracellular, glycosylphosphatidylinositol (GPI)-anchored enzyme (30, 31). If Car4 is the CO₂ sensor in the taste system, then (i) pharmacological block of extracellular carbonic anhydrases should abolish CO₂ taste responses, and (ii) a knockout of Car4 should selectively affect CO₂ taste detection. We examined nerve responses in the presence of benzolamide, a membrane impermeant inhibitor of carbonic anhydrase (32, 33) (see fig. S2). As predicted, gustatory (chorda tympani nerve) responses to CO₂ were highly susceptible to carbonic anhydrase inhibition (Fig. 3). Next, we characterized *Car4*^{-/-} mutant mice (32). Gustatory responses to CO₂ were indeed severely reduced in the mutants at all concentrations tested, whereas

Fig. 4. Requirement of PKD2L1-sour cells for the taste of carbonation. Representative integrated chorda tympani responses to sour, sweet, and CO₂ stimuli in wild-type mice or in animals expressing TeNT in PKD2L1 sour-sensing TRCs. See fig. S5 for responses to additional tastants and quantitative analysis.



responses to other taste stimuli, including sour, were unaltered. Thus, Car4 functions selectively as the main CO₂ sensor in the taste system.

Given that CO₂ taste sensing is completely eliminated in the absence of PKD2L1-expressing cells, we wondered why there are residual taste responses to CO₂ in the *Car4*^{-/-} animals. We hypothesized that the activity of additional carbonic anhydrases in these cells might provide the remaining activity. Indeed, dorzolamide, a broadly acting, membrane-permeable CA blocker (33) (see

fig. S2) abolished the residual gustatory responses to CO₂, even at the highest CO₂ concentrations tested (Fig. 3). Together, these studies strongly substantiate carbonic anhydrase as the CO₂ receptor, and support a mechanism in which the products of Car4 activity at the extracellular surface of TRCs (i.e., HCO₃⁻ and H⁺) are the principal salient stimuli for detection of carbonation.

How does CO₂ activate the taste system? Bicarbonate does not stimulate TRCs (fig. S3); thus pointing to protons as the relevant signal. Each of

the basic taste modalities is mediated by distinct TRCs, with taste at the periphery proposed to be encoded via labeled lines [i.e., a sweet line, a sour line, a bitter line, etc. (21)]. Given that Car4 is specifically tethered to the surface of sour-sensing cells, and thus ideally poised to provide a highly localized acid signal to the sour TRCs, we reasoned that carbonation might be sensed through activation of the sour-labeled line. A prediction of this postulate is that prevention of sour cell activation should eliminate CO₂ detection, even in the presence of wild-type Car4 function. To test this hypothesis, we engineered animals in which the activation of nerve fibers innervating sour-sensing cells was blocked by preventing neurotransmitter release from the PKD2L1-expressing TRCs. In essence, we transgenically targeted expression of tetanus toxin light chain [TeNT, an endopeptidase that removes an essential component of the synaptic machinery (34–36)] to sour-sensing TRCs, and then monitored the physiological responses of these mice to sweet, sour, bitter, salty, umami and CO₂ stimulation. As predicted, taste responses to sour stimuli were selectively and completely abolished, whereas responses to sweet, bitter, salty and umami tastants remained unaltered (Fig. 4 and fig. S5). However, these animals also displayed a complete loss of taste responses to CO₂ even though they still expressed Car4 on the surface of PKD2L1 cells. Together, these results implicate the extracellular generation of protons, rather than intracellular acidification (15), as the primary signal that mediates the taste of CO₂, and demonstrate that sour cells not only provide the membrane anchor for Car4 but also serve as the cellular sensors for carbonation.

Why do animals need CO₂ sensing? CO₂ detection could have evolved as a mechanism to recognize CO₂-producing sources (18, 37)—for instance, to avoid fermenting foods. This view would be consistent with the recent discovery of a specialized CO₂ taste detection in insects where it mediates robust innate taste behaviors (38). Alternatively, Car4 may be important to maintain the pH balance within taste buds, and might gratuitously function as a detector for carbonation only as an accidental consequence. Although CO₂ activates the sour-sensing cells, it does not simply taste sour to humans. CO₂ (like acid) acts not only on the taste system but also in other orosensory pathways, including robust stimulation of the somatosensory system (17, 22); thus, the final percept of carbonation is likely to be a combination of multiple sensory inputs. Nonetheless, the “fizz” and “tingle” of heavily carbonated water is often likened to mild acid stimulation of the tongue, and in some cultures seltzer is even named for its salient sour taste (e.g., saurer Sprudel or Sauerwasser).

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Supporting Online Material

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Materials and Methods

Figs. S1 to S5

References

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Sequential Processing of Lexical, Grammatical, and Phonological Information Within Broca's Area

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Words, grammar, and phonology are linguistically distinct, yet their neural substrates are difficult to distinguish in macroscopic brain regions. We investigated whether they can be separated in time and space at the circuit level using intracranial electrophysiology (ICE), namely by recording local field potentials from populations of neurons using electrodes implanted in language-related brain regions while people read words verbatim or grammatically inflected them (present/past or singular/plural). Neighboring probes within Broca's area revealed distinct neuronal activity for lexical (~200 milliseconds), grammatical (~320 milliseconds), and phonological (~450 milliseconds) processing, identically for nouns and verbs, in a region activated in the same patients and task in functional magnetic resonance imaging. This suggests that a linguistic processing sequence predicted on computational grounds is implemented in the brain in fine-grained spatiotemporally patterned activity.

Within cognitive neuroscience, language is understood far less well than sensation, memory, or motor control, because language has no animal homologs, and methods appropriate to humans [functional magnetic resonance imaging (fMRI), studies of brain-damaged patients, and scalp-recorded potentials]

are far coarser in space or time than the underlying causal events in neural circuitry. Moreover, language involves several kinds of abstract information (lexical, grammatical, and phonological) that are difficult to manipulate independently. This has left a gap in understanding between the computational structure of language suggested by linguistics and the neural circuitry that implements language processing. We narrow this gap using a technique with high spatial, temporal, and physiological resolution and a task that distinguishes three components of linguistic computation.

According to linguistic analyses, the ability to identify words, combine them grammatically, and articulate their sounds involves several kinds of

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representations, with logical dependencies among them (1, 2). For example, to pronounce a verb in a sentence, one must determine the appropriate tense given the intended meaning and syntactic context (e.g., “walk,” “walks,” “walked,” or “walking”). One must identify the particular verb, which specifies whether to use a regular (e.g., “walked”) or irregular (e.g., “went”) form. In addition, one must unpack the phonological content of the verb and suffix to implement three more computations: phonological adjustments in the sequence of phonemes (e.g., inserting a vowel between verb and

suffix in “patted” but not in “walked”), phonetic adjustments in the pronunciation of the phonemes (such as the difference between the “d” in “walked” and “jogged”), and conversion of the phoneme sequence into articulatory motor commands.

This logical decomposition does not entail that each kind of representation corresponds to a distinct stage or circuit in the brain. In many neural-network models, the selection of tense, discrimination of regular from irregular inflection, and formulation of the phonetic output are computed in parallel and in one time-step within a single distributed

network (3, 4). Others contain loops and feedback connections, propagate probabilistic constraints, and iteratively settle into a globally stable state, with no fixed sequence of operations (5). Even stage models may incorporate cascades where partial information from one stage begins to feed the next before its computation is complete (6). Nonetheless, the most comprehensive model of speech production, developed by Levelt, Roelofs, and Meyer (LRM), maximizes parsimony and falsifiability by implementing linguistic operations as discrete ordered stages, eschewing feedback, loops, parallelism, or cascades (7). They posit stages for lexical retrieval (which they associate with the left middle temporal gyrus at 150 to 225 ms after stimulus presentation), grammatical encoding (locus and duration unknown), phonological retrieval (posterior temporal lobe, 200 to 400 ms), phonological and phonetic processing (Broca’s area, 400 to 600 ms), self-monitoring (superior temporal lobe, beginning at 275 to 400 ms but highly variable in duration), and articulation (motor cortex) (8, 9).

Current evidence, however, leaves considerable uncertainty about the localization and timing of these components, especially grammatical processing. Although clinical studies report double dissociations in which a patient is more impaired in grammar than phonology or vice versa (10), in most studies both abilities are linked to similar regions in the left inferior prefrontal cortex, particularly Broca’s area (11). Although Broca’s area itself has been identified as the seat of phonology, grammar, and even specific grammatical operations (12–14), lesion and neuroimaging

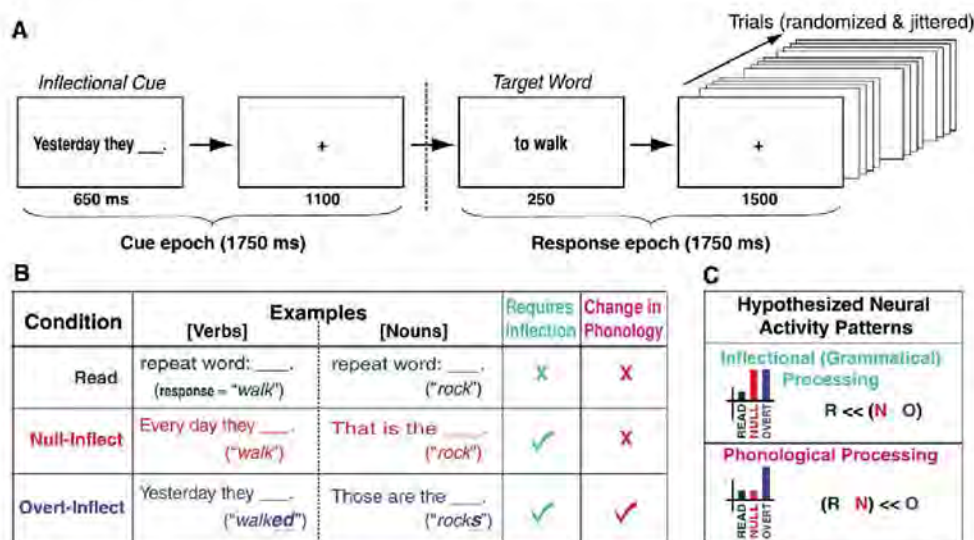
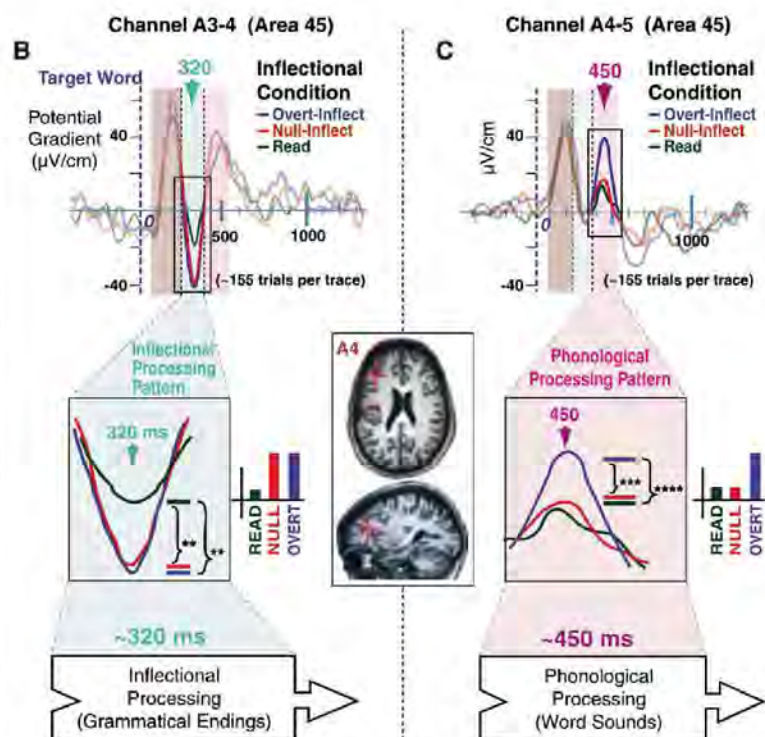
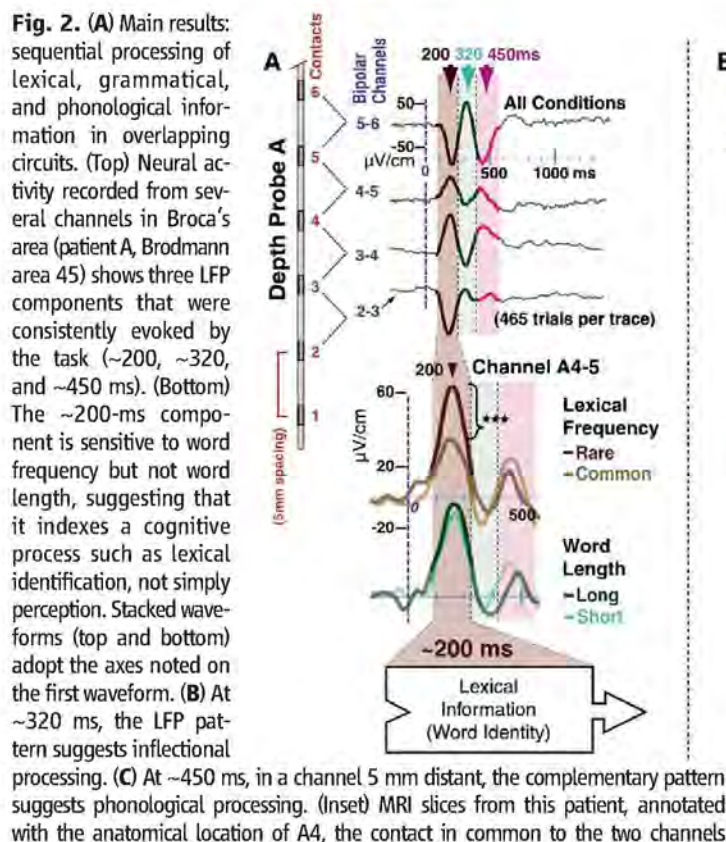


Fig. 1. Experimental design. (A) Structure of trials. (B) Experimental conditions, example trials, and required psycholinguistic processes. (C) Hypothesized patterns of neural activity by condition, for inflectional and phonological processing.



reported here. Statistical significance: **** ($P < .0001$), *** ($P < .001$), ** ($P < .01$) (t test, one tail, two-sample, equal variance). Box arrows (bottom) indicate linguistic processing stages, which may be interposed among other stages not addressed here.

studies have tied it to a broad variety of linguistic and nonlinguistic processes (15). This uncertainty may be a consequence of the coarseness of current measurements. It remains possible that grammatical and other linguistic processes are processed distinctly, even sequentially, in the microcircuitry of the brain, but techniques that sum over seconds and centimeters necessarily blur them.

In a rare procedure, electrodes are implanted in the brains of patients with epilepsy for clinical evaluation. Recordings of intracranial electrophysiology (ICE) from unaffected brain tissue during periods of normal activity can provide millisecond resolution in time with millimeter resolution in space. We recorded local field potentials (LFP) from multicontact depth electrodes in three right-handed patients (ages 38 to 51, with above-average language and cognitive skills) whose electrodes were located in and around Broca's area while they read words verbatim or converted them to an inflected form (past/present or singular/plural) (Figs 1 and 2) (16). The task engages inflectional morphology, which is like syntax in combining meaningful elements accord-

ing to grammatical rules, but the units are shorter and semantically simpler, making fewer demands on working memory and conceptual integration, and thus allowing greater experimental control. We applied the high resolution of ICE to a task that distinguishes three linguistic processes to investigate the spatiotemporal patterning of word production in the brain.

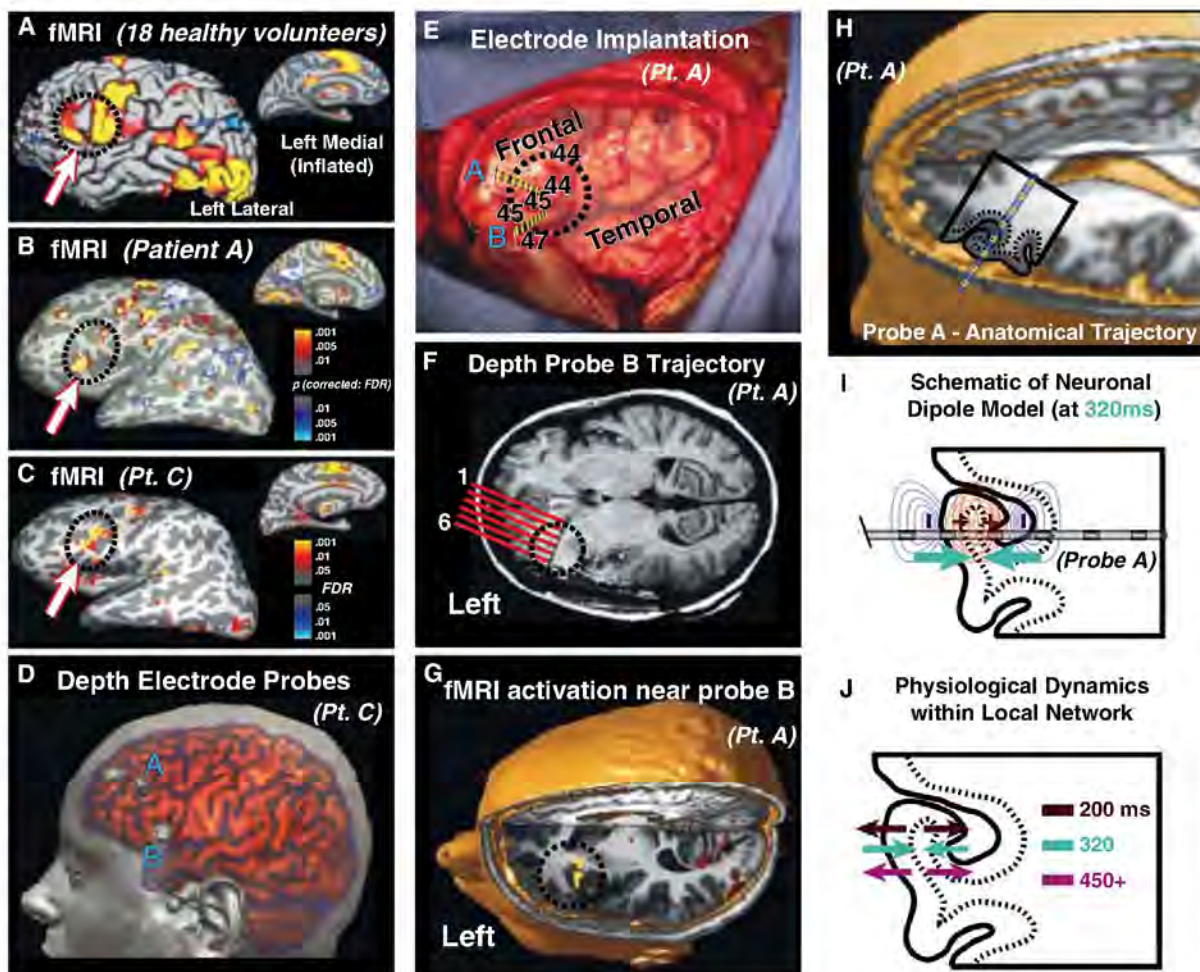
In each trial, participants saw either the instruction "Repeat word" (the "Read" condition) or a cue that dictated an inflected form ("Every day they ____"; "Yesterday they ____"; "That is a ____"; "Those are the ____"). Next, they saw a target word and produced the appropriate form silently (Fig. 1A) (16). The 240 target words were presented in uninflected form in the phrase "a [noun]" or "to [verb]" (17) (Fig. 1B). Half the targets were regular (e.g., "link"/"linked") and half irregular (e.g., "think"/"thought"), to ensure that participants had to access the word rather than automatically appending the regular suffix (18).

The Null-Inflect (N) condition requires an inflected form of the verb (present tense) or noun (singular), yet these forms are not overtly marked

and thus require the same output to be pronounced as in the Read (R) condition. The difference between these conditions thus implicates the process of inflection. In contrast, the Overt-Inflect (O) condition (past-tense verb or plural noun) requires that a suffix be added (regular) or the form changed (irregular). It thus differs from the Null-Inflect condition in requiring computation of a different phonological output (Fig. 1B). (The label "phonological" subsumes phonological, phonetic, and articulatory processes.) The design was fully crossed, with trials presented in pseudorandom order.

To assess whether these patients' language systems were organized normally, and to correlate LFP with fMRI, we performed fMRI in two of the patients before their electrodes were placed. Their activation patterns were indeed similar to 18 healthy controls (Fig. 3, A to C) [for other fMRI results, see (19)]. Most of the 168 bipolar channels from which we recorded (across patients) were in fMRI-active regions (Fig. 3, A to G). LFP that was significantly correlated with the task ($P < .001$, corrected) [see (16)] was recorded in about half (86 of 168) of the channels (19 channels in

Fig. 3. Localization of fMRI responses, depth electrodes, and neural generators. (A) fMRI in 18 controls, contrasting activity for all task conditions with visual-fixation baseline periods. The task engages classic language areas (Broca's, speech-related motor cortex, medial supplementary motor area, anterior cingulate, and superior temporal lobe) and visual-reading areas (visual word form area and primary and ventral visual cortex). Classic Broca's area is circled. Thresholding and correction at a 0.01 false discovery rate (16). Scale as in (B). (B and C) Single-patient fMRI (identical contrast) reveals similar activations in both patients and controls. Surfaces are inflated to reveal activation within sulci. (D) Coregistered MRI and computerized tomography scan of patient C showing depth probes inserted through the skull. (E) Intra-operative photo showing left perisylvian language areas. Letters, insertion points of the probes; dashed lines, surface projections of their intracortical trajectories. Putative Brodmann areas are labeled. (F) Postimplantation MRI reveals that probe B traverses Broca's area in the posteromedial process of IFG pars opercularis facing the insula, and preimplantation fMRI (G) demonstrates that the region was activated by the



task in this patient. (H) Location of probe A, in Broca's area traversing IFG pars triangularis within the inferior frontal sulcus. (I and J) Schematic of neural dipoles near probe A that generated the LFP components, hypothesized from their polarities, amplitudes, and locations (see fig. S3). Schematic gyral outline corresponds to the gyral trace superimposed on the MRI in (H).

patient A, 37 in B, and 30 in C). Of these channels, 49 (57%) were within Broca's area or the anterior temporal lobes (16 in patient A, 19 in B, 14 in C). Of the 49 channels, 26 were within Broca's area, and the majority (20 of 26) yielded a strong triphasic (three-component) LFP waveform (9 in patient A, 8 in B, 3 in C). The mean peaks occurred ~200, ~320, and ~450 ms after the target word onset (Fig. 2A), and this timing was consistent across patients (Fig. 4, A and B, and figs. S1, S4, and S5).

The three LFP components showed signatures of distinct linguistic processing stages (Fig. 2, A to C). The ~200-ms component appears to reflect lexical identification. The timing converges with when word-specific activity has previously been recorded in the visual word form area (VWFA) [(20, 21), but see (22)] and when the VWFA has been shown to become phase-locked with Broca's area (23). Furthermore, the magnitude of the component varied with word frequency, which indexes lexical access (24). Specifically, rare words (frequency 1 to 4) yielded a significantly higher amplitude [$t(204) = 3.32$, $P < 0.001$] than common words (frequency 9 to 12) (Fig. 2A) (25). Word frequency is inversely correlated with word length, but the present effect is not a consequence of length: We found no difference at ~200 ms between short (2 to 4 characters) and long (6 to

11 characters) words (Fig. 2A), nor a difference between one-morpheme and two-morpheme responses (26). Later components were not affected by frequency. Finally, consistent with the fact that lexical identification is required by all three inflectional conditions, the ~200-ms component did not vary across them. Primary lexical access is generally associated with temporal cortex rather than Broca's area (8), so this component may index delivery of word identity information into Broca's area for subsequent processing, consistent with anatomic and physiological evidence that the two areas are integrated (23, 27). Although word-evoked activity in this latency range has previously been localized to Broca's area with LFP (28) and magnetoencephalography (29), it has not been demonstrated to be modulated by lexical frequency.

The subsequent two LFP components showed activity patterns predicted for grammatical and phonological processing, respectively (Fig. 2, B and C). In the ~320-ms component (Fig. 2B), the Overt-Inflect and Null-Inflect conditions significantly differed from the Read condition but not from each other. Thus, the ~320-ms component is modulated by the demands of inflection (required by Overt-Inflect and Null-Inflect but not Read), but not by the demands of phonological programming (required in Overt-Inflect but not in Null-Inflect or Read;

see Fig. 1C). In contrast, in a component appearing at ~450 ms, Overt-Inflect did differ from the Null-Inflect and Read conditions, which did not differ from each other (Fig. 2C). This contrasting pattern indicates that the ~450-ms component reflects phonological, phonetic, and articulatory programming, independently confirmed by its sensitivity to the number of syllables (Fig. 4C). Both components were recorded from Broca's area in all patients (fig. S1), and specifically in patient A (Fig. 2) from the inferior frontal gyrus (IFG) pars triangularis deep in the inferior frontal sulcus. The ~320-ms component was recorded near the fundus; the ~450-ms component was recorded 5 mm more lateral along the sulcus within a subgyral fold that faced the fundus (Fig. 3I and fig. S1A). This region is often considered part of area 45 [but see (30)].

The pattern of sign inversions across neighboring bipolar channels in space (Fig. 2A, top) indicates that the generators of the LFP components were local (fig. S3), and the differences in inversions across components in time indicate that their generators were not identical (Fig. 3, I and J). Thus, the overall LFP pattern suggests a fine-grain spatiotemporal progression of lexical, grammatical, and phonological processing within Broca's area during word production.

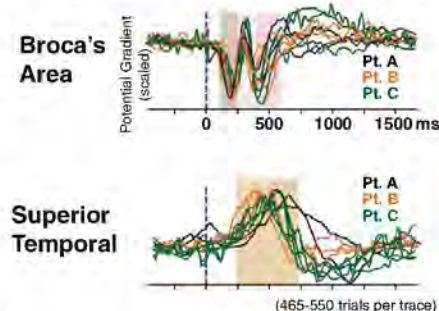
The triphasic pattern in all patients was found exclusively in Broca's area (Fig. 4A). Outside Broca's area, other patterns prevailed; for example, temporal lobe sites showed a slow and late monophasic component at 500 to 600 ms (Fig. 4A, bottom, and fig. S4, F and G) (31), possibly reflecting self-monitoring (7, 8). The condition differences for each component were also consistent across patients, replicating the temporal isolation of grammatical (~320 ms) from phonological (~450 ms) processing (fig. S1). The word-frequency effect on the ~200-ms component was significant in patients A and B and marginal ($P = 0.06$) in patient C (fig. S2). The ~200-, ~320-, and ~450-ms components were consistent in their timing across patients, although the keypress reaction times, which require the self-monitoring process, varied among patients and conditions (fig. S6).

Although nouns and verbs differ linguistically and neurobiologically (32, 33), the neuronal activity they evoked was similar (Fig. 4B). Furthermore, the patterning across inflectional conditions was the same for nouns and verbs (34). These parallels suggest that words from different lexical classes feed a common process for inflection.

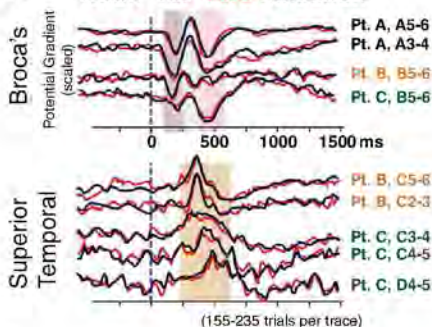
Additional evidence that the LFP patterns reflect inflectional computation is that they are triggered by presentation of the target word, not the cue, even though the cues contain more visual and linguistic elements (Fig. 4D) (35). Furthermore, activity evoked by the cue showed little sensitivity to the inflectional conditions.

The LFP patterns are consistent with the computational nature of the task and with independent estimates of the timing of its subprocesses. Inflectional processing cannot occur before the word

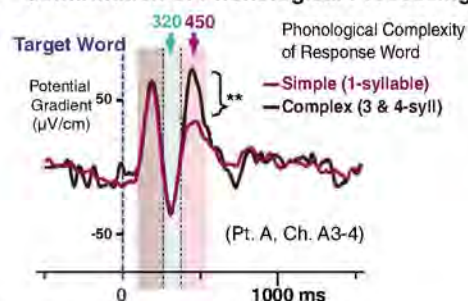
A Regional Specificity of Triphasic LFP



B Noun vs. Verb Inflection



C Confirmation of Phonological Processing



D Cue Epoch vs. Response Epoch

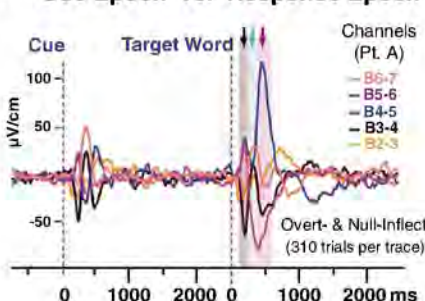


Fig. 4. Additional features of the triphasic waveform support the lexical-inflectional-phonological progression. **(A)** Triphasic activity is specific to Broca's area and is consistent across patients. All-condition average waveforms from task-active channels in each patient are superimposed (scaled in amplitude to a single channel in each region and standardized in polarity). **(B)** Noun (black) and verb (red) inflection (Null and Overt combined) involved nearly identical neural activity, across sites and patients. Standardized across channels in polarity. **(C)** The ~450-ms component, which is sensitive to phonological differences among inflectional conditions, is also sensitive to phonological complexity (syllable count) of the target word ($P < 0.01$, corrected). **(D)** Neural activity in Broca's area is evoked primarily when processing the target word (when the linguistic processing of interest should occur), not the cue (35).

is identified (especially as to whether it is regular or irregular), and phonological, phonetic, and articulatory processing cannot be computed before the phonemes of the inflected form have been determined. Word identification has been shown to occur at 170 to 250 ms (8, 29, 36), consistent with the ~200-ms component, and syllabification and other phonological processes at 400 to 600 ms, consistent with the phonological component at 400 to 500 ms (8). In naming tasks, speech onset occurs at around 600 ms (8), which is consistent with the self-monitoring behavioral responses we recorded (fig. S6). Self-monitoring has been localized to the temporal lobe (8), where we recorded LFPs in the post-response latency range that may correspond to previously described scalp event-related potentials (37). Working backward from 600 ms, we note that motor neuron commands occur 50 to 100 ms before speech, placing them just after the phonological component we found to peak at 400 to 500 ms (38). In sum, the location, behavioral correlates, and timing of the components of neuronal activity in Broca's area suggest that they embody, respectively, lexical identification (~200 ms), grammatical inflection (~320 ms), and phonological processing (~450 ms) in the production of nouns and verbs alike.

Although the language processing stream as a whole surely exhibits parallelism, feedback, and interactivity, the current results support parsimony-based models such as LRM (7), in which one portion of this stream consists of spatiotemporally distinct processes corresponding to levels of linguistic computation. Among the processes identified by these higher-resolution data is grammatical computation, which has been elusive in previous, coarser-grained investigations. As such, the results are also consistent with recent proposals that Broca's area is not dedicated to a single kind of linguistic representation but is differentiated into adjacent but distinct circuits that process phonological, grammatical, and lexical information (37, 39–41).

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Supporting Online Material

www.sciencemag.org/content/full/326/5951/445/DC1
Materials and Methods

Figs. S1 to S6

Tables S1 and S2

References

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Fast Synaptic Subcortical Control of Hippocampal Circuits

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Cortical information processing is under state-dependent control of subcortical neuromodulatory systems. Although this modulatory effect is thought to be mediated mainly by slow nonsynaptic metabotropic receptors, other mechanisms, such as direct synaptic transmission, are possible. Yet, it is currently unknown if any such form of subcortical control exists. Here, we present direct evidence of a strong, spatiotemporally precise excitatory input from an ascending neuromodulatory center. Selective stimulation of serotonergic median raphe neurons produced a rapid activation of hippocampal interneurons. At the network level, this subcortical drive was manifested as a pattern of effective disinhibitory GABAergic inhibition that spread throughout the circuit. This form of subcortical network regulation should be incorporated into current concepts of normal and pathological cortical function.

Subcortical monoaminergic systems are thought to modulate target cortical networks on a slow time scale of hundreds of milliseconds to seconds corresponding to the duration of metabotropic receptor signaling (1). Among these ascending systems, the serotonergic raphe-hippocampal (RH) pathway that primarily originates within the midbrain median raphe nucleus (MnR) is a key modulator of hippocampal mnemonic functions (2). Contrary to the slow

modulatory effect commonly associated with ascending systems, electrical stimulation of the RH pathway produces a rapid and robust modulation of hippocampal electroencephalographic activity (3–5). Anatomical evidence shows that MnR projections form some classical synapses onto GABAergic interneurons (INs) in the hippocampus (6), potentially providing a substrate for a fast neuromodulation of the hippocampal circuit. Recent reports of the presence of glutamate

in the serotonergic system (7–9) raise the possibility that this system may use a coordinated action of serotonin [5-hydroxytryptamine (5-HT)] and glutamate to rapidly activate elements of the hippocampal network.

To investigate this form of ascending neuromodulation, we first performed a series of *in vitro* experiments. Because RH axons are intermingled with intra- and extrahippocampal axons of various origins, we virally targeted a channelrhodopsin2-enhanced green fluorescent fusion protein construct (ChR2-eGFP) to the MnR (fig. S2, a to e) and subsequently used ChR2-assisted photostimulation in acute rat hippocampal slices to selectively activate the labeled RH input (10, 11).

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eGFP-positive (eGFP+) fibers formed a dense plexus mostly immunoreactive for 5-HT ($85 \pm 4\%$ 5-HT in eGFP+ RH fibers and $37 \pm 3\%$ eGFP+ in 5-HT+ RH fibers; $n = 3$ animals, fig. S2f) (11) in strata oriens/alveus and at the border of strata radiatum and lacunosum-moleculare (sr/sl-m) of CA1 and CA3 regions, matching previously documented laminar distribution of RH fibers (6, 12). We therefore focused on INs located in these areas to perform whole-cell voltage recordings. Two-photon imaging revealed that eGFP+ RH axons often formed what appeared to be climbing-type contacts at multiple close appositions with IN dendrites (Figs. 1A and 2A). To functionally characterize these putative contacts, we photostimulated the ChR2-eGFP+ axons using brief light exposures. Single light pulses (1 ms) evoked large-amplitude excitatory postsynaptic potentials (EPSPs) in most recorded INs (amplitude: 6.47 ± 1.33 mV, $n = 22$ out of 27 INs; Fig. 1, B and C). In many cases, this input was sufficient to trigger action potential output from the activated cell ($n = 7$ out

of 22; Fig. 2, A and B). Light-evoked EPSPs had a short latency (2.67 ± 0.17 ms, $n = 22$), small temporal jitter (SD of latency: 0.42 ± 0.07 ms, $n = 22$), a fast rise time, and a mono-exponential decay (20 to 80 rise time: 3.15 ± 0.31 ms; decay tau: 105.71 ± 10.79 ms, $n = 22$; Fig. 1, B and C). Repetitive photostimulation reliably evoked trains of EPSPs in INs (Fig. 1, D and E). Single-pulse photostimulation did not evoke detectable responses in CA3 pyramidal cells (CA3PC, $n = 9$ cells) and produced either no response (25 out of 29 cells) or only a slow, small hyperpolarization in CA1 pyramidal cells (CA1PC, peak hyperpolarization; single pulse: 0.31 ± 0.18 mV, $n = 4$; train: 0.91 ± 0.13 mV, $n = 21$; Fig. 1D).

To directly test whether EPSPs on INs are mediated by synapses formed by RH afferents, we accomplished correlated light and electron microscopic analysis of six appositions between eGFP+ and neurochemically identified boutons and recorded INs. Five of six boutons were glutamatergic, as indicated by positive immunoreactivity for vesicular glutamate transporter type 3

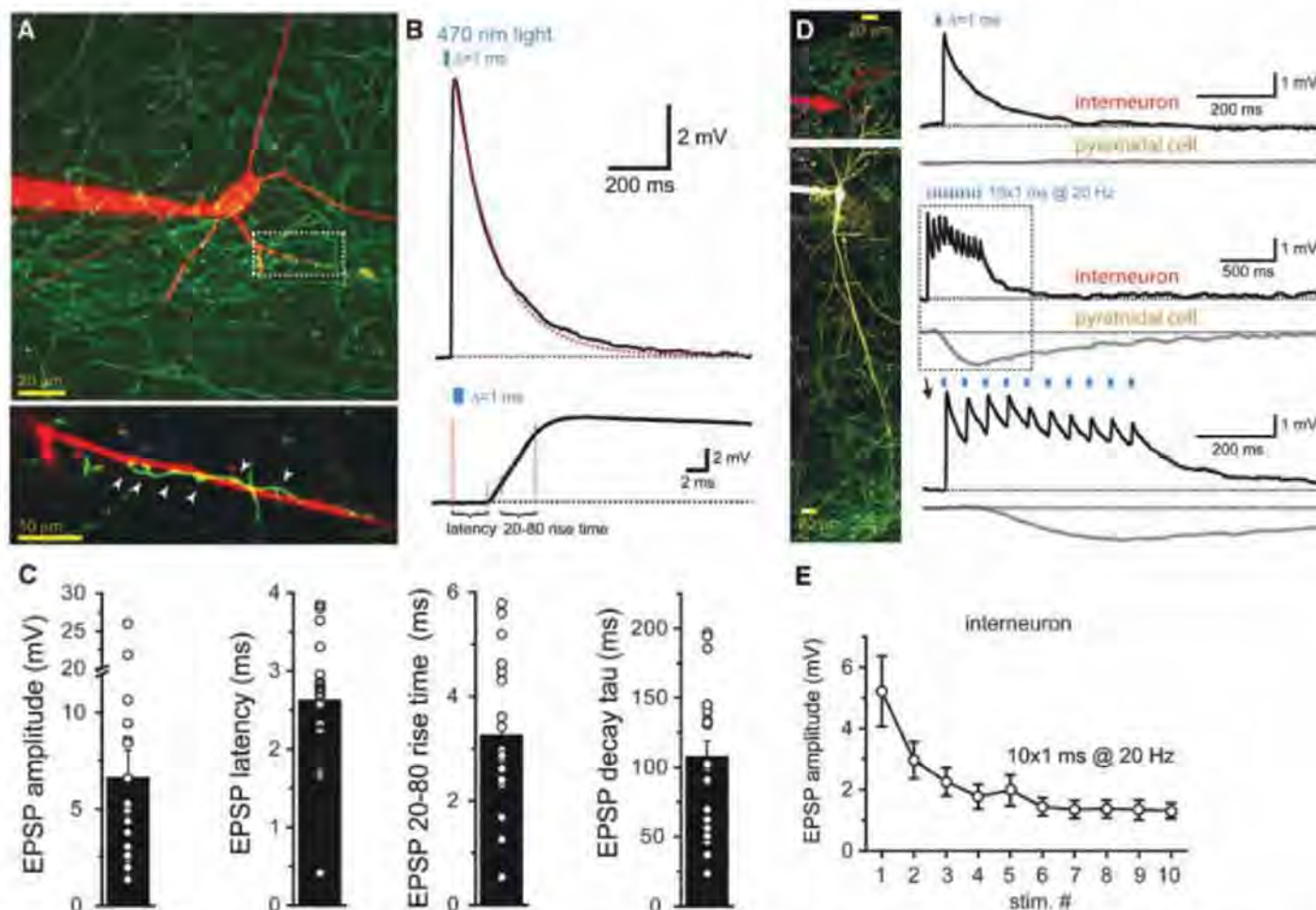


Fig. 1. MnR input excites hippocampal INs *in vitro*. (A) Two-photon image of an IN filled with Alexa 594 (red) surrounded by a dense network of eGFP+ RH fibers (green) at the border of sr/sl-m of CA1 region in a dorsal hippocampal slice. Dashed boxed region is expanded (lower panel) to show multiple putative contacts (arrowheads) made by an eGFP+ axon on the IN's dendrite. (B) (Upper panel) ChR2-photostimulation produces a large-amplitude and short-latency EPSP in the IN (red dotted line is exponential fit). (Lower panel)

Locations used to measure EPSP kinetics are indicated on an expanded time scale of the rising phase of the EPSP. (C) Summary data of EPSP kinetics. Symbols: individual recordings; bars: mean $\pm$ SEM. (D) Image of an IN (red) and a CA1PC (yellow-white) recorded sequentially (left) and averaged responses recorded from the IN (black) and from the PC (gray) to ChR2-photostimulation (right). (E) Summary plot of peak EPSP amplitude during train photostimulation from INs (10 pulses at 20 Hz, $n = 10$; error bars: SEM).

(VGluT3), and one was 5-HT/VGluT3 double immunoreactive ($n = 2$ INs, two connections tested for 5-HT/VGluT3 and four tested only for VGluT3). All examined appositions were synaptic contacts between the identified boutons and dendritic profiles of INs (Fig. 2, C to J, and fig. S3, a to d).

What type of neurotransmitters mediate fast transmission at RH-IN synapses? First, we tested the contribution of ionotropic 5-HT₃ receptors (5-HT₃Rs) that are expressed by a subset of hippocampal INs, which are innervated by MnR afferents (6, 13). The selective 5-HT₃R antagonist MDL72222 (100 μ M) significantly reduced the amplitude of the EPSPs (to $74 \pm 5\%$ of control, $P < 0.05$, $n = 16$; Fig. 3, A to D) (11). A prominent 5-HT₃R antagonist-resistant component was still present and was abolished by subsequent coapplication of AMPA-type (NBQX, 5 μ M) and *N*-methyl-D-aspartate (NMDA)-type (D,L-AP5, 50 μ M) ionotropic glutamate receptor (iGluR) antagonists [to $6 \pm 1\%$, $P < 0.001$, $n = 16$; the slow hyperpolarization on PCs was sensitive to 5-HT_{1A}R antagonist (S)-WAY 100135, 100 μ M; fig. S3e].

Two lines of evidence further support the dual-component serotonergic/glutamatergic nature of fast synaptic transmission in RH-IN synapses. VGluT3+ terminals at the border of sr/sl-m in the hippocampus CA1 area originate from raphe nuclei (8). Using electron microscopy (EM) and combined immunogold-immunoperoxidase staining, we found that most (19 out of 25) synapses

of these above-mentioned terminals and many (10 out of 16) synapses of RH terminals labeled by the anterograde tracer Phaseolus vulgaris leucoagglutinin (PHAL) are GluR1 subunit positive as well (Fig. 3, E to L). Moreover, in several cases, two-photon uncaging of MNI-glutamate (14) at putative synaptic contact sites made by eGFP+ axons evoked fast rising and decaying EPSPs in INs (fig. S3, f to i).

MnR activation may therefore robustly engage hippocampal INs via a fast serotonergic/glutamatergic excitation. To directly test this prediction, we first juxtacellularly recorded hippocampal neurons during electrical stimulation of the MnR in vivo ($n = 60$ cells; 8 cells were labeled and recovered, 4 cells were anatomically identified) (11). We found a subset of neurons that MnR stimulation activated with a short latency (8.9 ± 0.58 ms, $n = 16$; Fig. 4) (11). These fast activated cells showed a robust (firing: $491.2 \pm 155.2\%$ of control; success rate: $65.7 \pm 5.3\%$; Fig. 4, figs. S4 to S6, and table S2) and temporally focused activation (duration: 8.05 ± 1.03 ms; jitter: 1.7 ± 0.19 ms). All of these parameters except success rate were significantly different from those of a second subset of neurons that were activated more slowly (latency: 15 to 50 ms; $n = 7$, $P < 0.05$; table S2).

Evidence of the prominent glutamatergic component of the MnR-stimulation-mediated excitatory drive was also found in vivo. Local iontophoresis of NBQX ($n = 6$; table S3) (11) significantly suppressed the success rate of the

fast activation (2 min: to $53.8 \pm 4.5\%$; $P < 0.05$, $n = 6$; 5 min: to $29.7 \pm 11\%$, $n = 3$; Fig. 4, H and I) in a dose-dependent and reversible way, without altering the latency and nonsignificantly increasing the jitter of the response (table S3) (11). We determined the position of fast responding INs in the hippocampal network by identifying labeled cells immunocytochemically and analyzing their state-dependent activity (table S1 and figs. S4 to S6).

That hippocampal INs are highly interconnected (15) raises the possibility that the activated components of the inhibitory circuit could, through feed-forward inhibition, themselves play a role in producing the elevated spike precision described above. Further analysis of our MnR-stimulation data revealed that the initial increase in IN spiking was often followed by a period of suppressed activity (by $90.6 \pm 4\%$, 10 out of 16 cells; latency: 15 ± 1.71 ms; duration: 144.85 ± 17.32 ms; fig. 4F). Two further observations indicated that this postexcitatory suppression was caused by MnR-triggered inhibition. First, this effect appeared to be sensitive to 5-HT₃R antagonists, as ondansetron (1 to 2 mg/kg, intraperitoneal injection) significantly reduced the duration of the inhibitory period (from 148.5 ± 32.85 ms to 83.25 ± 14.13 ms; $n = 5$; 5 to 15 min after injection, $P < 0.05$) and increased the jitter of the primary facilitatory response (fig. S9h and table S3).

Photostimulation experiments were used to investigate this network effect in vitro. With a

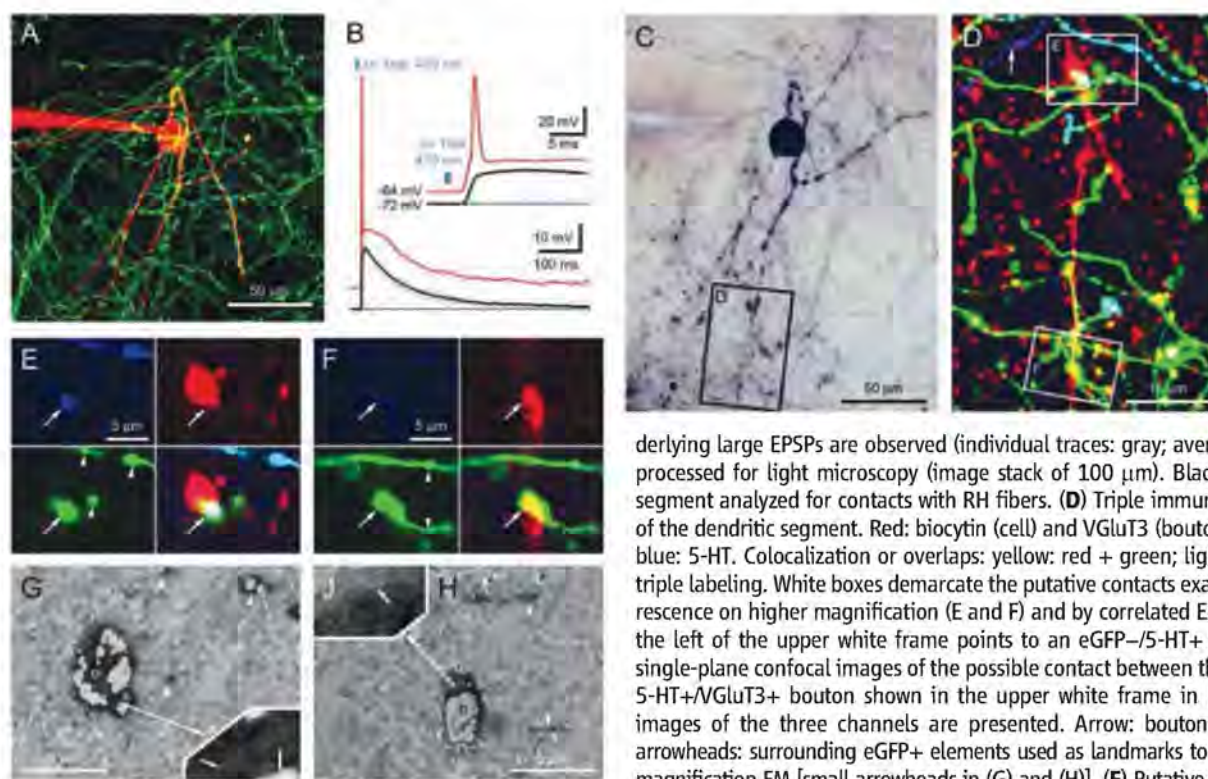
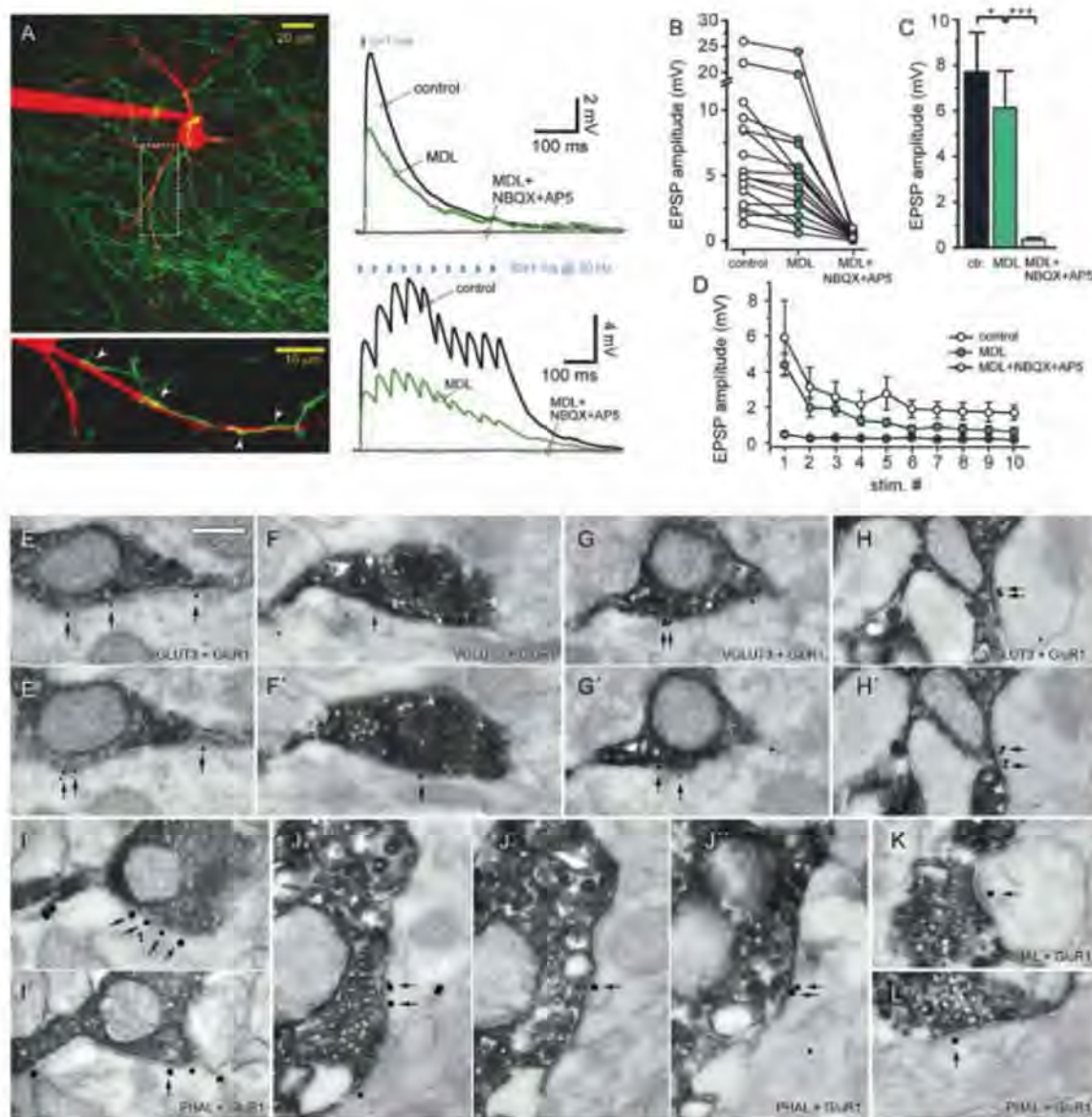


Fig. 2. RH fibers excite hippocampal INs via 5-HT- and/or VGluT3-containing synapses. (A) Two-photon image of an IN (red) and eGFP+ RH axons (green) at the border of sr/sl-m. (B) ChR2 photostimulation evokes short-latency action potential in the IN (red trace, right: expanded time scale) at resting membrane potential (V_m). At a more hyperpolarized V_m , underlying large EPSPs are observed (individual traces: gray; average: black). (C) The IN in (A) processed for light microscopy (image stack of 100 μ m). Black frame marks the dendritic segment analyzed for contacts with RH fibers. (D) Triple immunofluorescent confocal image of the dendritic segment. Red: biocytin (cell) and VGluT3 (boutons); green: eGFP (RH axons); blue: 5-HT. Colocalization or overlaps: yellow: red + green; light blue: blue + green; white: triple labeling. White boxes demarcate the putative contacts examined by triple immunofluorescence on higher magnification (E and F) and by correlated EM (G to J). The small arrow to the left of the upper white frame points to an eGFP-/5-HT+ fiber. (E) High-magnification single-plane confocal images of the possible contact between the dendritic segment and the 5-HT+/VGluT3+ bouton shown in the upper white frame in (D). Separate and overlaid images of the three channels are presented. Arrow: bouton examined by EM (G and I); arrowheads: surrounding eGFP+ elements used as landmarks to identify the area on the low-magnification EM [small arrowheads in (G) and (H)]. (F) Putative contact between the dendritic segment and the 5-HT- bouton designated by the lower frame in (D). (G) Low-power EM image of the contact depicted in (E). (H) Low-power EM image of the contact in (F). (I) High-power EM image [scale bar of (G) is 1 μ m in (I)] of the contact in (G) showing the parallel apposition of the dendritic and bouton membranes surrounding a possible synaptic cleft (small white arrow). The 3,3-diaminobenzidine tetrahydrochloride (DAB) precipitate masks synaptic vesicles. (J) The same as in (I) for the 5-HT-/VGluT3+ bouton shown in (H) [scale bar of (H) is 1 μ m in (J)]. B: RH boutons; D: IN dendrite.

segment and the 5-HT- bouton designated by the lower frame in (D). (G) Low-power EM image of the contact depicted in (E). (H) Low-power EM image of the contact in (F). (I) High-power EM image [scale bar of (G) is 1 μ m in (I)] of the contact in (G) showing the parallel apposition of the dendritic and bouton membranes surrounding a possible synaptic cleft (small white arrow). The 3,3-diaminobenzidine tetrahydrochloride (DAB) precipitate masks synaptic vesicles. (J) The same as in (I) for the 5-HT-/VGluT3+ bouton shown in (H) [scale bar of (H) is 1 μ m in (J)]. B: RH boutons; D: IN dendrite.

Fig. 3. Monosynaptic excitation of INs is mediated by 5-HT₃ and AMPA/NMDA receptors. **(A)** Upper left: Image of an IN (red) and eGFP+ RH fibers (green). Lower left: Expanded image of the dashed boxed region in upper panel; arrowheads indicate the positions of multiple close appositions between a proximal dendritic segment of the IN and eGFP+ axons. Traces to the right of the image are averaged V_m responses evoked by single-pulse and train photostimulation in control (black), MDL72222 (100 μ M, green), and MDL72222+NBQX+AP5 (gray). Subsequent coapplication of iGluR antagonists (NBQX 5 μ M, α -AP5 50 μ M) abolishes photostimulation-evoked responses. **(B and C)** Grouped data (B) and summary graph (C; error bars: SEM; * P < 0.05; *** P < 0.001) of EPSPs evoked by single-pulse photostimulation in INs. Lines connect individual recordings in control (open circles), MDL72222 (green circles), and MDL72222+NBQX+AP5 (gray circles). **(D)** Summary plot of peak EPSP amplitude during train photostimulation (n = 10; error bars: SEM). **(E to L)** EM images of immunoperoxidase-immunogold stainings. Images show GluR1 receptor subunit labeling in the synaptic contacts (arrows) that are established either by VGLUT3+ boutons [(E) to (H), DAB-positive dark precipitation] or by PHAL-labeled [(I) to (L), DAB-positive] RH terminals at the border of sr/sl-m. (E to J) Two or three sections of the same synapse with multiple labelings for GluR1 are shown. Scale bar on (E) applies for (F) to (L).



low concentration of chloride ($[Cl^-]_{in}$) in the intracellular solutions, monosynaptic EPSPs were often followed by readily observable GABAergic (i.e., gabazine-sensitive, n = 2) inhibitory postsynaptic potentials (IPSPs; fig. S9, a and b) (11). When present, these IPSPs were preferentially blocked by the 5-HT₃R antagonist (n = 5 out of 13 INs; fig. S9, b, e, and f). The most likely interpretation of this result (and observed in 7 out of 13 INs; fig. S9, c to f) is that the recorded IN is itself innervated by a subset of INs that are strongly excited by RH afferents. In agreement with this, IPSPs were disynaptic in origin, as the evoked responses were abolished by the coapplication of 5-HT₃R and iGluR antagonists (amplitude: to $7 \pm 2\%$, P < 0.05, n = 13; fig. S9, e to g).

Finally, we found that MnR activation of the inhibitory circuit is effectively transmitted onto the output elements, i.e., principal cells of the hippocampal circuit. When recording from PCs

with low $[Cl^-]_{in}$ in the intracellular solution in vitro, we observed robust disynaptic GABAergic IPSPs (fig. S10, a to c). This fast inhibition was also observed in PCs in vivo as a form of short-latency cessation of PCs spiking when recorded juxtacellularly (fig. S10d; n = 3) and as a short-latency hyperpolarization when recorded intracellularly (fig. S10e) during MnR stimulation (11).

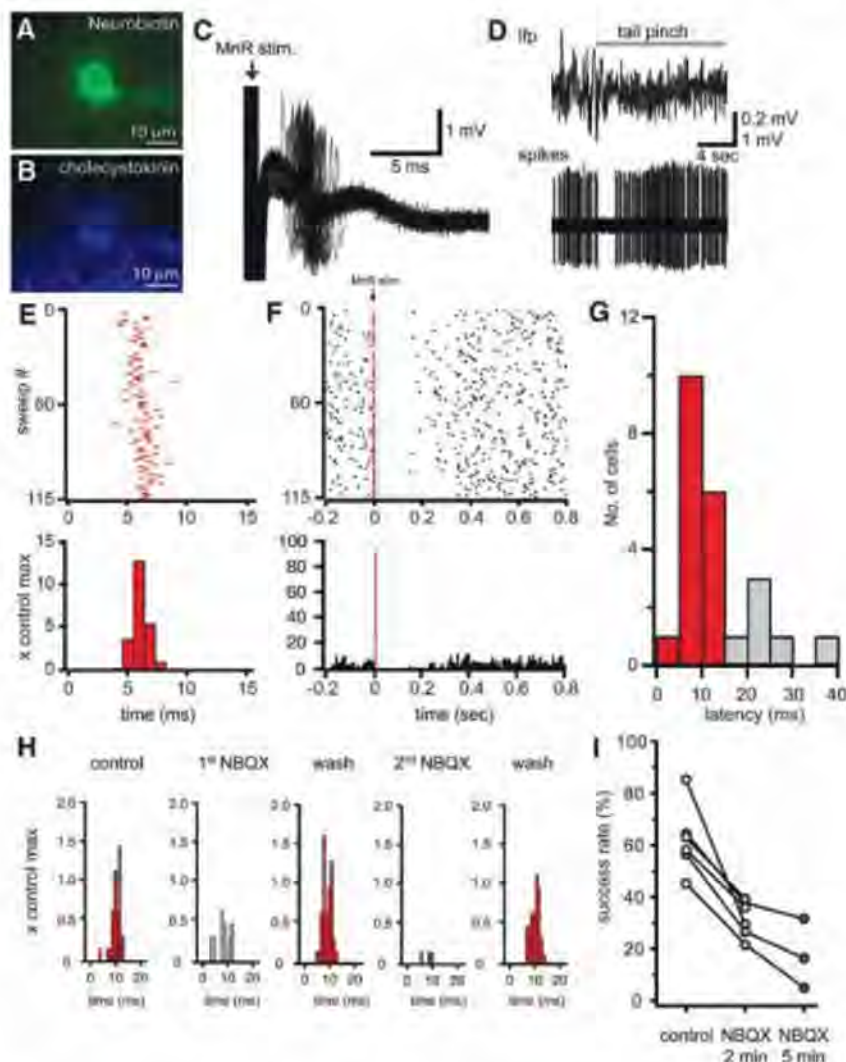
The present demonstration of a fast synaptic activation of hippocampal interneurons by MnR afferents via glutamate/serotonin cotransmission puts the subcortical control of cortical information processing in a fundamentally new perspective. The current view of a slow and diffuse modulation conveying emotional, motivational, or other state-dependent tuning (1, 16) is now complemented by the ability to carry out target-selective synaptic actions with high temporal and spatial resolution. This additional ability may promote the rapid formation and selection of particular

hippocampal local representations or modes of information processing (17, 18), possibly through fast alterations in the relative contribution of the different classes of interneurons to rhythmic population activity (13, 19) (fig. S1). Finally, the present observations lend support to a proposed role of glutamate in disorders that have traditionally been considered to be subcortical in origin, such as depression, anxiety, and certain components of schizophrenia (20–22).

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Fig. 4. A subset of hippocampal INs is activated by MnR stimulation *in vivo* with short latency by a partly glutamatergic mechanism. **(A and B)** Immunofluorescent identification of a fast-activated IN [(A) neurobiotin: green; (B) cholecystokinin: blue]. **(C)** MnR stimulation-induced activation of this IN is manifested as the concentration of evoked spikes within 10 ms of stimulation (overlying 20-ms windows of raw data, $n = 115$). **(D)** Firing pattern of the IN during the non-theta to theta transition. **(E and F)** Response of this IN is characterized by raster plots (upper) and peristimulus time histograms (PSTHs) (lower panels, PSTH bin size = 1 ms) showing the MnR stimulation-induced fast activation (E) and the subsequent long-lasting inhibition [(F, bin size = 10 ms)]. Note the robust increase in spike count (13 times that of the control, 6-ms latency). **(G)** Distribution of response latencies of neurons activated by MnR stimulation within 50 ms after stimulus onset ($n = 23$). The peak of fast responders can be clearly distinguished ($n = 17$ in red). **(H)** The fast response is largely attenuated by local application of NBQX: Doubling the duration of NBQX application (1st: 2 min; 2nd: 5 min) almost totally attenuates the fast response. After switching off NBQX ejection, the fast response recovers (1st and 2nd wash out). **(I)** Change of success rate of fast activation during all NBQX experiments (2 min, $n = 6$; 5 min, $n = 3$) after continuous drug iontophoresis. NBQX reduced the success rate in all instances, and its effect was time (dose) dependent.



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experiments. V.V. conducted the *in vivo* physiological experiments with the help of A.D. B.V.Z. performed the molecular experiments. A.L. performed the *in vitro* experiments. Z. B. and G.N. performed most of the histological experiments. N.H. performed the *in vitro* experiments for the specificity of the NBQX effect. V.V. and A.L. analyzed most of the physiological data except firing pattern analysis carried out by B.H. A.L., V.V., B.V.Z., J.C.M., and T.F.F. wrote the paper.

Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S10

Tables S1 to S3

References

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RNAi Therapeutics

RNAi THERAPEUTICS:

A TWO-YEAR UPDATE

Two years is a long time in the world of RNAi therapeutics. Since *Science* last covered the topic in 2007, the first Phase 3 human clinical trial of an siRNA drug has been prematurely terminated; new off-target effects have been identified; and the first miRNA-based therapeutic entered clinical trials. Yet much remains the same, especially the biggest challenge: delivery. As researchers struggle to overcome these obstacles, optimism persists undiminished. **By Jeffrey M. Perkel**

Optimism over the potential for RNAi therapeutics is emphatic. Says Judy Lieberman, senior investigator at the Immune Disease Institute of **Harvard Medical School** in Boston, “I think they are potentially superdrugs.” Lieberman, who recently published a paper using intravaginal delivery of short-interfering RNAs (siRNA) to protect mice from herpes virus infection, is not, she says, “being Pollyanna-ish.”

“I am aware of the problems and the obstacles,” she admits. “To get to the superdrug will take a lot of hard work and dedicated development.”

Yet that doesn’t dim her enthusiasm. The fact is that siRNAs—the double-stranded effectors of RNA interference (RNAi)—can “be developed to silence any gene; can be developed incredibly fast; can have picomolar efficacy; [and] can have an effect that lasts for weeks.”

In the lab, they have become invaluable tools, enabling the easy genetic knockdown of any sequence. The problem with migrating to the clinic, she says, is still delivery. “We have to figure out how to get them into cells better.”

Work on that front is proceeding at a rapid clip, in both academia and industry, as a handful of clinical trials attest. At the same time, new problems have cropped up, including unanticipated immunological effects and impacts on endogenous RNA processing. Others are probing the potential of the field as a whole, for instance with so-called “single-stranded” RNAi strategies.

“It’s obvious from what we know about the biology of RNA interference and how we are able to use it as a tool that it has tremendous potential, if we can harness it,” says David Corey, professor of pharmacology at **University of Texas Southwestern Medical Center** in Dallas. “Good delivery tools will help us do that.”

DELIVERY, DELIVERY, DELIVERY

John Rossi, the Lidow Family Research Chair at the Beckman Research Institute of the **City of Hope** of Duarte, California, says the three biggest problems with RNAi therapeutics remain “delivery, delivery, and delivery.”

It is, in fact, a multidimensional problem—to achieve RNAi, systemically administered nucleic acids must survive in circulation long enough to reach their target tissue, enter the desired cells, “escape” their endosome and/or delivery packaging, and finally become incorporated into the RNA-induced silencing complex (RISC)—and researchers have advanced a number of solutions over the past two years.

Tekmira Pharmaceuticals’ SNALP (stable nucleic acid-lipid particle) technology is generating excitement in the community, at least if clinical trials and collaborations are any indication: The company, which has partnered with Roche and **Alnylam Pharmaceuticals** (which uses SNALP technology in its Phase 1 clinical trials of ALN-VSP02 for advanced liver cancer), in July initiated a Phase 1 clinical trial of its own ApoB SNALP for hypercholesterolemia.

continued >

“I think they are potentially superdrugs.”



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RNAi Therapeutics

According to president and CEO Mark Murray, SNALP particles “contain four distinct lipids, which play distinct roles in the pharmacokinetics and pharmacodynamics of the product.” Mark Kay, director of the program in human gene therapy at **Stanford University**, who is on the company’s scientific advisory board, calls the technology’s therapeutic window “really the best out there.” He adds, “The doses at which there are clinically relevant responses do not cause toxicity in animal studies.”

Daniel Anderson at the **Massachusetts Institute of Technology**, collaborating with Alnylam, has broadened the library of compounds that potentially can be incorporated into such systems. His team used combinatorial chemistry to generate “over 1,200 structurally diverse lipid-like molecules,” a subset of which could deliver nucleic acids to cultured cells, mouse, rat, and nonhuman primates—not to mention ovarian tumor xenografts in mice. “It really expanded the chemical space people tried,” says Anderson.

Others are investigating nonlipid-based approaches. Steven Dowdy’s lab at the **University of California, San Diego School of Medicine**, developed a “peptide-transduction domain”/double-stranded RNA-binding domain fusion protein that can both bind siRNA and cross cell membranes. According to Dowdy’s graduate student Bryan Meade, the resulting particles are nontoxic, internalized by macropinocytosis, and then apparently degraded intracellularly, releasing the siRNA. “We can see RNAi as fast as 6 hours after delivery, as measured by quantitative RT-PCR,” he says.

Meanwhile, at the **University of Massachusetts Medical School**, Michael Czech, professor and chair of molecular medicine, has developed what he calls, “to our knowledge, the only technology that has been published that allows oral delivery of siRNA.”

GeRPs—or beta-1,3-D-glucan-encapsulated siRNA particles—are “solid but porous shells composed almost entirely” of a specific yeast cell wall component, says Czech. Within the glucan shell, the particles wrap siRNA in a transfection reagent, cationic polyethyleneimine. Delivered orally to mice, the GeRPs can silence tumor necrosis factor- α expression in gut macrophages.

RNAi therapeutics firm **RXi Pharmaceuticals** is using GeRPs in its in-house development efforts, says Tod Woolf, president and CEO, as well as so-called “self-delivering rxRNAs”—chemically modified RNAs that can enter cells without a vehicle. Though he declines to describe the enabling chemistry, Woolf does say rxRNA technology involves three different kinds of modifications: “They make compounds bind cells, get taken up, and get released into cells.”

LOCAL VICTORIES

Companies have also had success with local delivery strategies.

Completed in spring 2008, Alnylam’s 88-patient Phase 2 GEMINI trial of ALN-RSV01, a naked, chemically modified siRNA delivered intranasally against respiratory syncytial virus (RSV), “provided the first human proof of principle results showing statistically significant antiviral efficacy [from RNAi],” says John Maraganore, CEO of Alnylam.

Specifically, ALN-RSV01 produced “an approximately 40 percent reduction in RSV infection rate and 95 percent increase in infection-free subjects,” according to a corporate statement. Data from a second, 24-patient Phase 2 trial, announced in July, indicate it too “achieved its primary objective of demonstrating safety and tolerability” over 90 days posttreatment. But, Maraganore clarified, “[That] study wasn’t powered to show activity.”

TransDerm, with the International Pachyonychia Congenita (PC) Con-

sortium and the PC Project, found smaller-scale success with a one-patient, placebo-controlled Phase 1b trial of TD101, a naked, unmodified siRNA for treatment of PC.

A painful genetic disorder, PC causes blisters and thickened skin on the soles of the foot, says company CEO Roger Kaspar; just to administer the drug intradermally required pain medication. Following injection, “we saw what appeared to be pink skin that was no longer tender to the touch,” Kaspar says.

However, as injections are especially problematic for PC patients, TransDerm is investigating other delivery options, including dissolvable microneedle arrays and even a skin cream dubbed “GeneCreme.”

With so many available options, it’s unlikely any one will dominate the RNAi landscape. “There’s not going to be a one-size-fits-all solution,” says Beverly Davidson, the Roy J. Carver Biomedical Research Chair of Internal Medicine at the **University of Iowa**, who pursues both naked and viral-mediated RNAi approaches to central nervous system disorders. “It’s more likely that one technology will be better for one application than another.”

THREE STEPS FORWARD, ONE STEP BACK

Unfortunately, as with any new science, unexpected roadblocks inevitably arise on the way to the clinic. “With every three steps forward, there is a step back,” Davidson says.

Christina Leslie of the **Memorial Sloan-Kettering Cancer Center** and Debora Marks of Harvard Medical School exposed one such setback this year when they showed that transfection of siRNA into cells tends to upregulate microRNA-controlled genes, suggesting that the exogenous nucleic acids can swamp intracellular RNA-processing machinery.

Jayakrishna Ambati’s lab exposed another when, in March 2008, they demonstrated that a naked siRNA, injected into the eye, could block angiogenesis in mouse models of age-related macular degeneration regardless of its sequence. Whether targeting vascular endothelial growth factor or green fluorescent protein, a random sequence or firefly luciferase, every siRNA seemed to block choroidal neovascularization, says Ambati, the Dr. E. Vernon Smith and Eloise C. Smith Endowed Chair at the **University of Kentucky**.

The findings seemed to fly in the face of accepted RNAi wisdom—and directly into the path of an ongoing human clinical trial. **Opko Health’s** bevasiranib, a naked siRNA targeting vascular endothelial growth factor for age-related macular degeneration (AMD), was on track to become the first FDA-approved siRNA therapeutic.

The drug had ridden into Phase 3 trials on the basis of animal data indicating it could block the subretinal vascularization that causes blindness in AMD. But those studies used a transfection reagent not used in the human trials. Ambati’s results suggested the drug’s efficacy had nothing to do with RNAi, but rather with an innate immune reaction to double-stranded RNAs mediated by the Toll-like receptor-3 (TLR3).

Ambati says the findings are not terribly surprising; researchers knew that in general, cells don’t take up naked siRNA. “It seems to me that there was an irrational exuberance about the technology, and to be frank, the science behind these [original bevasiranib animal studies] is not sound and contradicts very good science showing that double-stranded siRNAs don’t get into cells.”

That perception seemed vindicated March 6 when Opko Health, citing the recommendation of its Independent Data Monitoring Committee, announced it was terminating its Phase 3 trial of bevasiranib because “the trial, as structured, was unlikely to meet its primary continued >

RNAi Therapeutics

end point.”

But Phillip Sharp, Institute Professor at the Massachusetts Institute of Technology, cautions against drawing a straight line from Ambati's findings to Opko's announcement. “The TLR studies were interesting, but small RNAs interacting with TLRs had been described before,” he says. “That does not in any way qualify the utilization of small RNAs in clinical studies.”

For its part, Opko remains bullish on RNAi, says Jamie Freedman, executive vice president of research and development and business development. Though no siRNA trials are ongoing, the company has not abandoned bevasiranib, he says. It is, however, pursuing other delivery options, as well as other siRNA targets. But Opko also is not putting all its eggs in one basket; the company “is also expanding its portfolio with other kinds of therapeutics, both in and outside of ophthalmology.”

GOING SINGLE-STRANDED

Though researchers have identified ways to circumvent the TLR3 effect (using siRNAs shorter than 21 bases, for instance), just as they have ways to avoid miRNA swamping, it's also true that not all oligonucleotide structures elicit these responses.

Double-strand RNAs also present fundamentally different—and more complex—manufacturing and delivery challenges than single-strand molecules. “A double-stranded structure is structurally and functionally as different from a single strand as night and day,” says Isis CEO Stan Crooke. Rigid where a single strand is relatively flexible, hydrophilic rather than amphipathic, and with double the mass and volume of a single-stranded molecule, double-stranded RNAs—unlike their single-stranded counterparts—are not readily distributed to most organs in vivo, Crooke says.

For these reasons, some are pursuing single-strand RNAi strategies instead of double-stranded ones. The technique differs from traditional

antisense RNA, in that antisense RNA targets RNAs for degradation via RNase H, while RNAi does the same using RISC.

According to Crooke, that distinction is one of semantics, not science. “I don't believe the field has broadened. I believe RNAi researchers' understanding has broadened. siRNA is an antisense mechanism: The active moiety is the antisense strand, and the sense strand is a drug-delivery device.”

Developer of Vitravene, the first FDA-approved antisense therapeutic, Isis now appears poised for the agency's next oligonucleotide approval, as well: mipomersen. One of 19 drugs in Isis' pipeline, mipomersen is a “first-in-class” ApoB synthesis inhibitor “to reduce LDL-C in patients with high cholesterol and who have high cardiovascular risk.” In May the company disclosed Phase 3 clinical data of individuals with familial hypercholesterolemia indicating that the drug had “met its primary endpoint, with a 25 percent reduction in LDL cholesterol after 26 weeks of treatment, versus 3 percent for placebo.” According to Crooke, the company plans to file an NDA in 2010.

Regulus Therapeutics and **Santaris Pharma** are also pursuing antisense strategies, but directed at microRNAs rather than messenger RNAs.

“miRNA inhibition provides a way to affect multiple gene pathways that have evolved together and are regulated by the target miRNA,” says Henrik Ørum, Santaris's chief scientific officer. “This pathway modulation is fundamentally different from the single-gene approach achieved by targeting and reducing the expression of an mRNA.”

Santaris achieved an industry milestone in 2008 when it initiated a Phase 1 clinical trial of SPC3649, a locked nucleic acid-based antisense molecule targeting miR-122, a host RNA required for hepatitis C replication, and the first microRNA-targeted therapeutic to reach the clinic.

Regulus achieved a milestone of its own when it helped show the therapeutic benefits of antagonizing miR-21 in a mouse model of heart failure. According to Kleanthis Xanthopoulos, Regulus president and CEO, the study “demonstrates for the first time that inhibition of an overexpressed miRNA has a therapeutic effect in a mouse model of human disease.”

For Xanthopoulos, microRNA represents the “third column” of what he calls the “genus [of] ‘RNA therapeutics’” (the others being antisense RNA and RNAi). And, with the constant discovery of new roles and species of noncoding RNAs, that genus is growing—as is its potential. That's because unlike monoclonals, which can target only extracellular proteins, oligonucleotide therapeutics “can touch every single gene, and every miRNA,” Xanthopoulos says. It is an idea Maraganore calls, “drug-ging the undruggable genome,” and it could be huge. “If proteins and monoclonal antibodies are a \$40 billion industry, RNA therapeutics can be significantly higher,” says Xanthopoulos.

It won't happen overnight—it took two decades to turn monoclonals into an approved therapeutic—but with some two-dozen oligonucleotide compounds in clinical trials, including at least eight siRNAs (two each from Alnylam and Quark Pharmaceuticals, plus one each from Calando Pharmaceuticals, Tekmira, TransDerm, and Silence Therapeutics), and around 10,000 people having been dosed in those and previous trials, “We are not talking about potential, we are at a tipping point,” says Xanthopoulos.

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NEUROSCIENCE

“Our philosophy is that only through basic research can you undertake novel approaches to disease.”
—Morgan Sheng



Meanwhile, new neuroscience complexes are sprouting up elsewhere, including the Ernst Strüngmann Institute in Frankfurt, Germany, named after the founder of the drug company Hexal. Andreas and Thomas Strüngmann, his successors, donated more than \$300 million for this facility. This marks the first time Germany's Max Planck Society has used private money to establish a research institute, says **Wolf Singer**, director of the Max Planck Institute for Brain Research and interim head of the new institute. “We hope it will set a precedent for other public-private research ventures in Germany.”

The center will create jobs for about 80 to 100 scientists, according to Singer, plus another 40 people for infrastructure. Though the focus will remain flexible, a priority will be placed on primate research, he says. “Given how rare this has become in Europe because of animal research restrictions, my feeling is that once we lose that, it'll be gone forever.”

Much of what is known about nerve cell processing comes from single-cell recordings, Singer says. “But that's clearly not the end of the story. The brain is a highly distributed system that uses distributed codes that can only be understood if you look at many cells at the same time.” The coordination of these distributed functions in the brain leads to specific behaviors, he adds. “That's where animal models are essential. We can look at many nerve cells at the same time while simultaneously charting behavior.”

As the Strüngmann Institute is located on the university's medical campus, it will take advantage of the availability of patients to probe what Singer calls “the highest integrated functions” and their pathologies. Arguably the most convenient and least invasive way of doing that is through functional magnetic resonance imaging, or fMRI—a technique that measures changes in blood flow and blood oxygen levels in the brain, thereby showing which parts of the brain are activated when people perform various tasks. By virtue of this tool, researchers have gained insights on how healthy brains work, while also identifying signs of brain abnormality.

Consequently, the use of fMRI has exploded worldwide since its invention in the early 1990s. “The last time I checked, 12,000 papers had been published on fMRI-related research since 1992,” says Washington University in St. Louis neuroscientist **Marcus Raichle**. The number of papers reflects both the excitement at having an unprecedented window into the brain at work and the increased availability of machines themselves. Devices that were once found only in elite radiology centers are now scattered throughout university departments. “The majority of research today is done on machines dedicated to researchers,” Raichle notes.

At first, fMRI was used primarily to explore classical questions in-

volving attention, memory, language, and perception. Its use was later extended to the study of diseases like Alzheimer's, schizophrenia, autism, and stroke. A relatively new area is to look at the developing nervous system by scanning infants, children, and adults at rest, to chart changes in activity patterns as the brain matures. fMRI scanners also allow Raichle and other researchers to study the brain's “dark energy”—baseline activities that are unrelated to external stimuli or the performance of overtly visible tasks, yet consume the vast majority of the brain's energy. “It's fair to say that a large fraction of the brain's functional activity is unaccounted for,” notes Raichle, who's hoping fMRI can help unlock this mystery.

As with functional brain imaging, the emerging technology of neuroprosthesis is opening up many new doors in the field. A team led by Brown University neuroscientist John Donoghue has implanted sensors in the brains of four quadriplegic patients that connect signals from the motor cortex to output devices, thereby enabling paralyzed patients to move computer cursors, control robotic limbs, and operate wheelchairs. Parallel efforts are under way at many other universities—all sharing the common goal of restoring functions to people with disabilities. The endeavor is bringing new people into neuroscience, including those accomplished in electronics, signal processing, sensor design, and other aspects of engineering.

“The diverse activities now under way illustrate that there are many different ways of being a neuroscientist,” says **Claudia Kawas**, a neurologist at the University of California, Irvine. “I work on a population study with more than one thousand 90-year-olds. Others in my department are studying learning and memory in sea slugs (*aplysia*) and songbirds. There are people who rarely see anything other than a cell culture, and those who spend all day looking at computer screens. There are also those who put mice in mazes and genes in mice. There is, in other words, something for everybody.”

Kawas focuses on the aging brain, which is a growth area, partly owing to demographics: In much of the world today, she says, people over 90 comprise the fastest-growing segment continued »

Featured Participants

Brown University
www.brown.edu

Columbia University
www.columbia.edu

Eli Lilly and Company
www.lilly.com

Gatsby Charitable Foundation
www.gatsby.org.uk

Genentech
www.gene.com

Massachusetts Institute of Technology
www.mit.edu

Max Planck Institute
www.mpg.de/english

National Institute of Neurological Disorders and Stroke
www.ninds.nih.gov

Society for Neuroscience
www.sfn.org

The Wellcome Trust
www.wellcome.ac.uk

University College London
www.ucl.ac.uk

University of California, Irvine
www.uci.edu

Washington University in Saint Louis
www.wustl.edu

NEUROSCIENCE

"It's fair to say that a large fraction of the brain's functional activity is unaccounted for."

—Marcus Raichle



of the population. Though there are fewer than 2 million people in that category in the United States today, forecasts suggest there will be 10–12 million a few decades from now. Perhaps the biggest concerns for this portion of the population is dementia—including Alzheimer's, the most common form—and other kinds of cognitive impairment. While getting to the root of Alzheimer's has proved immensely challenging, there's the further mystery of "Disease X." Half the people over 90 who suffer from dementia have no obvious brain pathology, Kawas says. "It's hard to know how to treat these people because we still don't know what's wrong with them."

"When I started in this field 25 years ago," she adds, "someone said they'll cure Alzheimer's and then there will be nothing to do. I now know that will never happen. We won't run out of questions to explore. Alzheimer's, like cancer, is not a single disease, and no single cure will take care of it all."

Nevertheless, with tens of millions of people suffering from Alzheimer's worldwide, there is keen interest in finding medical treatments for this disease. And the same holds for Parkinson's, amyotrophic lateral sclerosis, and other neurodegenerative ailments.

Like much of the pharmaceutical industry, Eli Lilly and Company had focused its central nervous system drug development efforts on psychiatric illnesses, but is now making major forays into neurodegenerative diseases as well. "The need in this area is enormous and growing," says **David Bredt**, who oversees the company's neuroscience research. Lilly currently has two Alzheimer's drugs in Phase 3 clinical testing (the final phase before seeking FDA approval). One drug blocks an enzyme essential to the formation of amyloid plaques that are thought to cause the disease. The second drug is intended to clear away the peptides that constitute the principal components of the plaque. "We're getting a clearer understanding of the pathologies—amyloid plaques and their analog in Parkinson's, Lewy bodies—and how we can thwart their formation," says Bredt. Psychiatric illnesses like schizophrenia and depression are more mysterious, he adds, because there are no known brain lesions scientists can ascribe to those conditions.

"Neuroscience remains an area of tremendous unmet medical need," Bredt notes. "With the burden of disease dramatically increasing along with an aging population, it constitutes among the largest therapeutic markets for drugs."

Genentech, one of the world's biggest biotech companies (recently purchased by Roche), seems to agree with that assessment. The company put its neuroscience program on hold a decade ago but is now making a renewed push in this area. The time is right owing

to recent advances in understanding how the brain works, claims the company's neuroscience head **Morgan Sheng**, "coupled with the fact that most diseases of the nervous system are very inadequately treated."

Genentech is focusing on neurodegenerative conditions, with additional efforts in pain and psychiatric illness. The company, which employs more than 100 postdocs, places a premium on basic (as well as translational) research, encouraging its scientists to publish in top journals. "Our philosophy is that only through basic research can you undertake novel approaches to disease," Sheng says. "If you leave the basic research to academia, all you can do is react."

Sheng left a tenured position at MIT as a professor and Howard Hughes investigator, moving to Genentech last year in order to have "a more direct impact on human well-being. My decision relates to the prospect that in the next 10 years we can come up with treatments for brain disease that really help people," he says. "Obviously, you cannot fully do that in academia; you have to work with a drug company."

Nevertheless, Sheng believes the wall between academia and industry is not nearly so pronounced as it once was. "There's a lot of exchange between the two, with the flow going both ways," he says. "Moreover, the kind of people we're looking for—good scientists with solid training in multiple aspects of neuroscience—aren't really different from what academic institutions are looking for."

It is certainly the case that more career paths are open today, says **Thomas Carew**, president of the Society for Neuroscience (SfN) and chair of the University of California, Irvine's Department of Neurobiology and Behavior. "When I started out 30 years ago, there was typically just a single path, which led to university professor. While that's still the primary goal for many, there are other choices today." In addition to jobs in industry and journalism, exciting new subfields are opening up for example in law, where neuroethics is playing an important role, and neuroeducation, which involves educational strategies that attempt to capitalize on neuroscience insights.

"Students shouldn't try to figure out where the field is going," Carew says. "They should make decisions based on what excites them and where their passion lies." For those who are unclear as to what the possibilities may be, SfN—which has 38,000 members, a third of whom are graduate students and postdocs—offers an array of professional development programs that highlight various career choices.

One way of easing the transition from research fellow to working professional is through a National Institutes of Health grant. Pathway to Independence Awards, for instance, are specifically designed to smooth the transition from postdoc to independent research. Investigators with novel ideas, especially those who are in the early stages of their careers, might consider applying for New Innovator Awards. A variety of other funding sources can, of course, be pursued. "It's a spectacular time to be a neuroscientist," says **Story Landis**, a neuroscientist who heads the National Institute of Neurological Disorders and Stroke. "There are fantastic opportunities to do great science and really make a difference."

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